Project IV: Airway Surface Liquid Composition of Humans *In Vivo*

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DESCRIPTION. State the applications broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This description is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. DO NOT EXCEED THE **SPACE PROVIDED.**

The long-term goal of this project is to better define the role of normal airway epithelial ion (and water) transport in lung defense, and identify the primary (and other early) pathogenic events in CF lung disease. The overall hypothesis of this proposal is that the surface epithelia in normal airways exhibit "isotonic volume absorption", and the primary pathophysiologic event in CF lung disease involves accelerated absorption of isotonic liquid, which limits the normal hydration of mucus in conducting airways. Mucus stasis ensues, with impaction in small airways and predisposition to chronic bacterial infection. We will specifically address an alternative hypothesis, which suggests that normal airways absorb ions, but not volume, and generate a hypotonic airway surface liquid (ASL). Under the "hypotonic ASL" paradigm, the pathogenesis in CF airways is similar to that of the sweat duct, i.e., an inability to absorb NaCl, which results in higher concentrations of salt relative to normal, and inhibition of salt-sensitive small-peptide antimicrobial activities. We will use specific techniques *in vivo,* including ion-selective electrodes, to study upper and lower airway epithelial function and ASL composition. We will study normal subjects, CF patients (including infants and neonates), and pertinent disease-control groups, including patients with pseudohypoaldosteronism (PHA) and Sjögren's syndrome. These studies are designed to define the relative contributions of surface epithelia and SMGs to ASL metabolism, in part to test theories related to SMG dysfunction in the pathogenesis of CF airway disease. We will measure the water content (percent solids) of airway secretions in CF and normals to test the hypothesis that accelerated isotonic volume absorption leads to reduced hydration of mucus in CF. Pertinent data will also be generated that address the role of specific inflammatory or neutrophil functions in early pathogenesis. Finally, studies of BAL in CF infants and neonates will directly test whether mucus obstruction precedes infection, or whether infection occurs first.

PERFORMANCE SITE(S) *(organization, city, state)*

The University of North Carolina at Chapel Hill
Chapel Hill, North Carolina

a. SPECIFIC AIMS

The early pathogenesis of CF lung disease is not understood. Two hypotheses have been advanced to link abnormal CF ion transport to lung disease. The "isotonic volume absorption" hypothesis predicts that the primary pathophysiologic event in CF lung disease involves accelerated absorption (and/or defective secretion, e.g., from glands) of isotonic liquid. This abnormality limits normal hydration of mucus, which results in mucus stasis and impaction in small (distal) airways, and ultimately, chronic bacterial infection. An alternative hypothesis suggests that normal airways absorb ions, but not volume, and generate a hypotonic airway surface liquid (ASL). Under the "hypotonic ASL" paradigm, the airways dysfunction in CF is similar to that of the sweat duct, i.e., an inability to absorb NaC1, which results in higher concentrations of salt relative to normal, and inhibition of salt-sensitive small-peptide antimicrobial activities.

The overall goal of this proposal is to distinguish between these two major hypotheses, which embrace fundamentally different concepts of normal airway epithelial ion and liquid transport, and consequently predict very different effects on ion (and water) composition of ASL. To distinguish between these hypotheses, protocols will be performed that will measure the ionic and water content of ASL. Specific techniques will be used to make measurements of *in vivo* ASL composition in the upper and lower airways in normal subjects, CF patients, and pertinent disease control groups. Our studies will also encompass protocols to define the relative functions of surface epithelia and SMGs, to provide an integrated description of mucosal physiology, and to test theories related to SMG dysfunction and pathogenesis of CF lung disease. Finally, this Project will test whether mucus stasis/obstruction precedes infection in CF (predicted by "isotonic volume absorption" hypothesis) or whether infection precedes obstruction (predicted by "hypotonic ASL" hypothesis). Pertinent clinical data relevant to these hypotheses, and inflammatory and neutrophil responses, will be generated. Two Specific Aims are proposed to achieve these goals:

Specific Aim 1: Measure ionic composition of ASL in large and small airways of normal subjects, CF patients, and disease controls, and measure the relative contributions of surface epithelium and submucosal glands (SMGs) to the balance of airway surface liquid (ASL) ionic composition.

A. Studies of nasal mucosa using filter paper technique and ion-selective electrodes.
   1. Measure ASL ion composition under basal conditions.
   2. Characterize function of surface epithelium after inhibition of SMGs (ipratropium) with respect to ASL ion composition, water permeability, and rates of volume absorption.
   3. Characterize SMG function by measuring ion composition of stimulated SMG secretions.

B. Studies of lower airway epithelial function
   1. Measure ASL ionic composition in proximal bronchi (using filter papers and ion-selective electrodes) under basal conditions and after inhibition of SMG secretion.
   2. Measure ASL ionic composition in distal airways using ion-selective electrodes

Specific Aim 2: Measure the mass of "mucus" and its hydration in airways **of normal subjects, CF patients with a range of disease, and disease control subjects**

A. Studies of the nasal mucosa (in adolescent and adult subjects)
   1. Measure the water content and mass of mucus recovered under basal conditions by standardized collection techniques
   2. Determine the contribution of SMGs by measuring the water content (and mass) of mucus after stimulation of SMG secretion and collection by standardized techniques.

B. Studies in the lower airways of CF and "normal" and disease control neonates and children (< age 3) who are well-characterized with respect to inflammation and infection.
   1. Quantitate "total mucin" in BAL harvested by standardized techniques
   2. Characterize the cellular and macromolecular composition of "mucus plugs"
   3. Measure the state of hydration and ion content of "mucus plugs"

b. BACKGROUND/SIGNIFICANCE

Introduction: This proposal is designed to investigate the initial pathogenesis of airways disease in

CF. Although the pathogenesis of lung disease in CF is likely linked to defective ion transport, we do not understand the mechanism, in part because we do not understand how normal airway epithelial ion (and liquid) transport contributes to lung defense. We are missing quantitative data in normal airways regarding: 1) the balance of ion and liquid transport between surface and SMG epithelia; 2) the composition of ASL under different conditions; and 3) the interplay between ion (and liquid) transport and the hydration of the "mucus" gel that is critical to mucociliary clearance (MCC). An important component of this proposal is to measure ASL in normal subjects as well as CF patients because this information will discriminate between the two major hypotheses. In addition, we must test the prediction of each hypothesis on the progression of disease, specifically whether mucus plugging precedes inflammation or infection, or whether infection occurs first.

Hypotheses regarding early pathogenesis in CF: This proposal will focus on two fundamentally different concepts of normal physiology of ASL, and the consequences of deranged function in CF. The traditional concept, based on observations by Kilbum and early ion transport studies at UNC, suggests that isotonic volume transport occurs across normal airway surface epithelia, as the volume of ASL is moved by MCC from distal (large surface area) to proximal airways (much smaller surface area) (1-6). In CF, excessive isotonic volume absorption, driven by accelerated Na^+ transport, limits the volume of liquid available to hydrate airway mucins. In this hypothesis: 1) the ASL ion composition is isotonic under basal unperturbed conditions in normal and CF; 2) the hydration (water content) of mucus is decreased in CF; and 3) the initiating pathophysiologic event is abnormal mucus plugging in small airways, predisposing to infection.

The alternative (hypotonic) hypothesis, based on observations by Quinton and studies of airway bacterial killing activity at Iowa, suggests that normal airways maintain ion concentration gradients, i.e., absorb ions but not volume, and generate hypotonic surface liquid, which implies the epithelium is either water impermeable, or other forces (capillarity) retain water on airway surfaces (4,7-9). In CF, the inability to absorb NaCl (similar to sweat duct) results in ASL with a high salt concentration (relative to normal ASL), which inhibits salt-sensitive "defensin-like" activities (8,9). Under this hypothesis: 1) the ASL ionic strength should be higher in CF; 2) the hydration of mucus should be similar in normal and CF; and 3) the initiating pathophysiologic event should be bacterial infection in the absence of abnormal airway secretions.

Surface epithelial function in bronchi: Available information on surface epithelial ion (and volume) transport does not discriminate between the "isotonic volume absorption" versus the "hypotonic ASL" hypothesis for either normal or CF airways. The cross-sectional surface area of small (non-cartilagenous) airways is more than 4000-fold greater than proximal airways, which would obligate volume absorption (as much as 1,500 ml per day) if ASL volume (both gel and sol layer) is moved proximally by MCC (6). However, there are no data to show that most ASL moves along airway surfaces. In normal airways, Na^+ absorption is the dominant active ion transport. Under physiologic (open-circuit) conditions, the major counter-ion is Cl^-, which may be absorbed via cellular or paracellular pathways (2,10-13). However, there is no definitive link between Na^+ transport and volume absorption across normal airway epithelia. Reported rates of Na^+ transport-linked (amiloride-sensitive) volume absorption are highly variable in human airways epithelia, ranging from rates consistent with ion fluxes *(2-5* pi/cm^2 hr) to very low rates (0.1 ìl/cm^2·hr) (12,14,15). In CF airways, the dominant abnormality of ion transport is accelerated Na^+ absorption, which reflects the lack of inhibition of ENaC by mutated CFTR (3,11,16-23). Although the apical membrane of CF airway epithelia is impermeable to Cl^- under basal conditions, the rate of Cl^- absorption under physiologic (open-circuit) conditions in freshly-excised tissues is raised in CF (3,24), which probably reflects paracellular Cl^- movement (13,25,26). However, the reported rates of volume absorption across CF airways have varied. Jiang et al. (14) measured an increased rate of amiloride-sensitive volume absorption across CF epithelia, compatible with accelerated Na^+ absorption (3), whereas Smith et al. (22) report that CF epithelia absorb volume at a rate no different from normal. Further, absorption was not blocked by amiloride, suggesting a failure of CF airway epithelia to absorb Na^+ (Cl^-) from ASL (parallels the CF sweat duct). Clarification of the relationship between Na^+ transport and volume flow in CF airways is essential to understand early pathogenesis, and we will test that relationship with *in vivo* measures of ASL ion composition, and volume absorption (± amiloride).

Surface epithelial function in bronchioles: Bronchial epithelia do not secrete CF under baseline conditions (2,10,13,25), but it is not known whether aglandular bronchioles secrete Cl^- (liquid) as a source of

ASL, or whether alveolar liquid is propelled onto bronchiolar surfaces by surfactant gradients (27). The latter concept implies volume absorption, and available evidence supports that concept, i.e., cultured bronchiolar cells and intact animal bronchioles absorb Na^+ as the basal function (28-32). However, CFTR is expressed in surface epithelia of human bronchioles (33), which might normally be a source of ASL (Cl^- secretion). If so, a defect in CFTR-mediated Cl^- secretion could lead to a reduced volume of ASL on distal airway surfaces, and add to the defective hydration of mucins associated with excessive Na^+ absorption in proximal airways.

Role of submucosal glands (SMGs) in CF pathophysiology: The expression of CFTR in SMG serous cells, and descriptions of dilated SMG ducts as an early pulmonary lesion in CF infants, led to the concept that SMG dysfunction plays a role in CF airways disease (4,33-37). CFTR-mediated Cl^- secretion by serous cells is considered a major source of SMG liquid secretion, but Cl^- secretion is blunted in CF SMG seromucous cells compared to normal (3 8,39). If CFTR plays a critical role in SMG secretion, the defect in CF is predicted to reduce volume output and/or reduce secretion of normally abundant mucins and antimicrobials (lysozyme; lactoferrin) (40-44). However, we have not observed major alterations in volume output or ion composition in cholinergic-stimulated CF SMG secretions (see Prelim. Data), which suggests the presence of a non-CFTR pathway(s) for SMG secretion (45). Furthermore, in contrast to isotonic SMG secretion in cats (46), normal and CF subjects secrete a hypotonic SMG liquid (see Prelim. Data). This observation implies NaC1 absorption across a water impermeable SMG duct, which is congruent with parotid duct function in both groups (47,48).

Composition of airway surface liquid (ASL): Conducting airways are covered by ASL that contains ions, water, and macromolecules, organized we speculate as periciliary fluid and a "mucus" layer on top (1-3). Effective clearance by MCC requires a complex interaction among ciliary function and ASL volume and ionic composition. Differences in epithelial function and ASL composition have broad ramifications for airway defense. For example, salt-sensitive antimicrobials require very hypotonic *(*50 mM NaCl) ASL for optimal activity (8,9), whereas optimal ciliary transportability requires higher concentrations of NaC1 (49). The volume of ASL is critical to the formation of a mucus gel, which involves: 1) exchange of Na^+ (from the ASL) for Ca^{2+} inside the condensed mucin, and 2) swelling of the mucin network by "hydration" (50), which reflects not only the available volume but also the concentration of salt and pH (49-51). Available information on ASL ion composition does not discriminate between "isotonic volume absorption" versus "hypotonic ASL". Studies are limited because it is difficult to collect and measure samples of ASL. Proximal airway ASL of animals is mildly hypotonic to mildly hypertonic *(52-56).* Recent studies of normal and CF nasal and bronchial ASL using filter papers and Na^+-selective electrodes suggest that there is no difference in the tonicity or ion composition of ASL between normal subjects and uninfected CF patients, although the nasal ASL is isotonic and bronchial and SMG ASL is hypotonic (see Prelim. Data). These results contrast with one study showing raised Cl^- (170 mM) in CF ASL (mucopurulent secretions) (57), and another study showing hypotonic (~220 mEq/L) bronchial ASL in normals, but isotonic ASL in 3 CF patients and 8 non-CF patients with infection (58).

Early pathologic features of CF lung disease: There was early debate about infection as the initiating event in the lung, but pathological studies of CF neonates dying secondary to non-pulmonary complications demonstrated plugging of numerous small airways (including bronchioles) with stringy mucus secretion in neonates as young as three days in the absence of infection or inflammation (34-3 7). An early, extensive study "seemed conclusive.., that the essential change consists in... thick mucus... which accumulates in the air passages"(34). This concept spawned a research effort that has lasted more than four decades to study CF mucus, but no CF-specific abnormality in mucin has been identified (59).

Bronchoscopy studies to define early pathophysiology in CF: Recent data on infants and young children with CF indicate that many do not have detectable infection at the time of bronchoscopy, and within this "uninfected" group there are subgroups with and without inflammation (60-62). The uninfected, uninflamed subgroup was especially prominent (~50%) when very young infants were studied (61). Thus, there appears to be a short period in infancy and early childhood that precedes either chronic bacterial infection or inflammation. Many of the non-infected infants have small (<3 mm diameter) "mucus plugs" recovered by BAL, which supports the concept that abnormal airway secretions may precede infection. However, the cellular, macromolecular, and ionic constituents of these plugs have not been identified. Published studies (60-62) and recent BAL data (see Prelim. Data) suggest that bacterial infection is associated with excessive or prolonged

neutrophil-dominated inflammation in CF, which may involve diminished production of the anti-inflammatory cytokine IL- 10 by epithelial and immune cells or delayed apoptosis contributing to excessive neutrophils (63-67). Not reported is total antimicrobial activity [small (< 10 kd) and large (> 10 kd) molecular fractions] in the BAI of uninfected CF and control infants. We hypothesize that airway obstruction with abnormal secretions is the early abnormality predisposing to chronic infection, and thick secretions (mucus plugs) and/or changes in ion composition of ASL may adversely affect neutrophil function. Thus, a complete characterization of BAL relevant to early airway pathophysiology of CF should involve ASL ionic composition, quantity and state of hydration of mucus, antimicrobial activity, inflammation, and infection.

Pertinent disease controls: Two disease control groups will provide insight into airways pathogenesis in CF. Patients with systemic pseudohypoaldosteronism (PHA) have mutations in the epithelial Na^+ channel, and defective Na^+ absorption in multiple organs (68-70). We have demonstrated an absence of amiloride-sensitive Na^+ transport in the nasal epithelium of PHA patients, and we are characterizing the clinical status of these patients (in collaboration with E. Kerem, M.D., Hebrew University, Jerusalem, and E. MacLaughlin, M.D., U. of S. California). For this project, we will measure the volume and ion composition of nasal ASL in P1-IA patients. "Isotonic volume absorption" would predict that these patients would have excess, but isotonic, ASL, and follow a clinical course distinct from CF. In contrast, if the "hypotonic ASL" hypothesis is correct, the isotonic ASL in these patients should be associated with defective salt-sensitive antimicrobial activity, and CF-like lung disease. Sjögren's syndrome is an autoimmune disorder with lymphocyte-mediated destruction of exocrine glands, including SMGs in the respiratory tract (71). The dominant respiratory symptom is a dry cough, which has been ascribed to "dry airways", but MCC in these patients is normal (72). It is controversial whether these patients have an increased incidence of airway infections, but the course of the airways disease is clearly distinct from CF (71,72). We hypothesize that the nasal SMGs of Sjögren's patients will secrete poorly (or none) in response to cholinergic-stimulation, and the basal ASL composition will be dominated by surface epithelial function. This would be consistent with our major hypothesis, and against the notion that SMG dysfunction plays a major role in the pathogenesis of CF airways disease.

Summary: There are no published comprehensive studies on the ionic (and water) composition of ASL in normal humans. Our studies will focus on measuring ionic composition of ASL in the nose (readily accessible) and lower airways (site of disease pathogenesis) of normal subjects, CF patients, and disease controls to discriminate between different hypotheses. Collection of ASL is difficult and current techniques may modify the basal composition in lower airways. We will address these limitations by blocking SMGs (ipratropium), and new techniques (small ion-selective electrodes) to rapidly measure ion concentrations *in situ*. We shall proceed to test for a biologic correlate of the excessive isotonic volume absorption, i.e., the dehydration of mucus in CF airways as compared to normal. If the isotonic, volume absorbing hypothesis is correct, we should find more mucus in BAL due to poor clearance in CF. We will directly measure the hydration of nasal mucus, but the collection of mucus from distal airways is currently dependent on BAL with saline, which may alter the structure/hydration of plugs. Alternative lavage solutions are being explored, including non-electrolyte (glucose) and immiscible perfluorocarbons (73). Finally, we will measure variables that are relevant to other hypotheses of pathophysiology, i.e., SMG function and antimicrobial (> or < 10 kd) activity, as well as variables pertinent to the role of inflammation and inflammatory cells.

c. PRELIMINARY DATA

Specific Aim 1: Measure ionic composition of ASL in large and small airways of normal subjects, CF patients, and disease controls, and measure the relative contributions of surface epithelium and submucosal glands (SMGs) to the balance of airway surface liquid (ASL) ionic composition.

1A. Nasal surface liquid composition (filter paper technique)

The collection and measurement of the ion composition of small volumes of ASL sampled by the filter paper technique are subject to methodological errors, and substantial effort has been undertaken to validate techniques for weighing, extraction of electrolytes, and ionic analyses. (See Experimental Design and Methods and Analytic Core for technical details.) Regarding weighing, the first measurement of filter paper samples is

~15 sec after the sample is obtained; serial weights are performed over 90 sec, and a regression line is extrapolated to time 0 for the initial weight. The evaporative loss of weight of a known sample of saline (10 ìl) added to the filter paper was 3.1 ± 1.2% over the initial 15 sec. Back extrapolation from serial weighing gave an initial weight that was *99.5* ± 1.2% of the volume of saline added to the filter paper at time 0. Regarding extraction, electrolytes were eluted from the filter paper in acid-washed vials in 1 ml of doubled-distilled/deionized H_20 on a shaker for at least 24 hr. The solution was transferred to vials containing 0.2 N HNO_3 *(0.5* ml), and papers were re-washed with 0.2 N I-1$N0_3$ in a final volume of 2 ml. Extraction efficiency was tested by adding solutions (10 ìl and 25 ìl) of a known composition (Na^+, Cl^-, and K^+) to the filter papers; the mean recovery of these ions from the filter papers was 98.2 ± 1.8%. Regarding ionic analyses, we measured Cl^- on a chloridometer, and Na^+ and K^+ on a flame photometer. Standard curves were reproducible, and the estimated limit of detection was ~2.5 ìl of a solution (100 mM NaCl). Blanks were run in parallel, which comprise less than 4% of the value for any specimen.

We also tested our complete system by measuring the recovery of a defined electrolyte solution that was deposited on the nasal mucosa *in vivo*. After pretreatment of the nasal mucosa with ipratropium to inhibit gland secretion (74,75), nasal surfaces were sprayed with a NaCl (150 mM) solution. The ionic composition of surface liquid sampled —30 sec after the nasal spray from under the inferior turbinate with direct application of filter paper (20 sec) was similar to that of the saline spray (150 mM NaCl) for both normal and CF subjects. The K^+ was low *(~5* mM) compared to baseline ASL (see below), which suggests little cell damage by the filter paper. These data provide strong support for the sampling, elution, and ionic analyses to accurately measure the composition of liquid on the nasal surface.

Baseline composition of nasal surface liquid-(filter paper)

To address the problem of evaporative water loss from ASL, a series of experiments were performed to define the duration of time after occlusion of the nostrils for steady-state equilibration of ion concentrations to be achieved. The variability of the electrolyte concentrations (as well as the volume of liquid sampled) from nasal surfaces was high until the occlusion time approached 10 mm; values at 20 mm were not different in magnitude or variability than at 10 mm. Summary data of composition of nasal ASL from normal and CF subjects after 10 mm of nasal occlusion are shown (Fig. 1). The weights collected by filter paper from normal and CF subjects were not statistically different (18.0 ± 2.2 mg and 24.0 ± 3.8 mg, respectively), although the samples from the CF patients tended to have more thick, "mucus-gel" type secretion adherent to the filter papers. The ion composition of ASL from normal subjects had a Na^+ concentration ~25% below that of plasma, whereas the K^+ concentration was 5-fold greater than plasma. The concentration of Cl^- was slightly higher than plasma, and the calculated anion gap (i.e., $Na^+ \pm K^+$ - Cl^-; an estimate of the putative HCO; concentration) was less (12 mEq/L) than the concentration of HCO; in plasma. Estimated osmolarity of the nasal surface liquid [2 (Na^+ + K^+)] was close to isotonic (estimated ~277 mOsm/L) with plasma. Importantly, the ionic composition (Na^+, Cl^-, K^+) of samples from CF subjects was not different from normal.

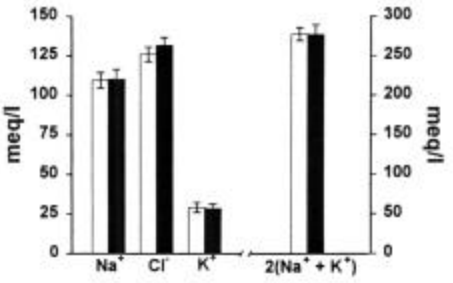

Figure 1. Basal ionic composition and estimated osmolarity of nasal ASL in normal subjects (n=8; open bars) and CF patients (n=8; solid bars) after 10 min of nasal occlusion.

Nasal ASL composition after SMG blockade with ipratropium (filter paper)

The composition of ASL at baseline (Fig. 1) reflects the net balance between SMG secretion and surface epithelial ion transport. To assess the role of the surface epithelium, SMG secretion was blocked with an ipratropium spray (168 µg in 150 mM NaCl) (74,75). After 20-40 minutes, the nose was occluded for 10 mm and nasal surface liquid from under the inferior turbinate was sampled by filter papers (Fig. 2). The samples were ~50% smaller than at baseline in both normal and CF subjects; again, the CF samples tended to be larger because of adherent "mucoid-type" secretions. The estimated osmolarity (265-280 mEq/L) from both groups was similar to baseline values (~isotonic), and the Na^+ and K^+ concentrations for CF and normal were similar to baseline values. However, there was a striking difference in the Cl^- concentration; the Cl^- in normals was lower (105.2 mEq/L) than baseline (126.0 mEq/L) and the Cl^- in CF was higher (139.9 mEq/L) than baseline (131.8 mEq/L). These changes in Cl^- concentration were reflected in the anion gap, i.e., the anion gap in normals increased from baseline (12 to 27 mEq/L), and the gap decreased in CF (6 to 0.1 mEq/L). In the absence of SMG secretion, the surface epithelia in normals appear to absorb Cl^- with Na^+, but the CF surface epithelia appear to absorb another anion (?HCO_3^-) with Na^+ in preference to Cl^-. These data imply that the tonicity of ASL is not different between CF and normal, but surface epithelia in CF modifies ASL ion composition differently than normal. In future experiments, it will be critical to see if the mucus on CF nasal surfaces is dehydrated, i.e., measure the % dry wgt (i.e., state of hydration, % water), and whether the pH is different, i.e., measure HCO_3^-/pH of these secretions.

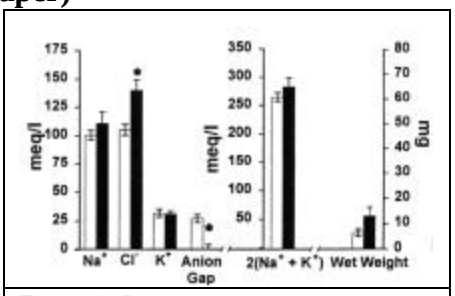

Figure 2. Ionic composition, osmolarity, anion gap, and wgt of sample of nasal ASL after nasal ipratropium to block SMG secretion in normal subjects (n=6; open bars) and CF patients (n7; closed bars). **p<O.05* - different from normal.

ASL composition after stimulation of SMG secretion (filter paper)

We sought to define the contribution of SMG secretion to the composition of ASL using gustatory (chili peppers) stimulation of nasal SMGs (76). The nose was occluded for 10 mm, hot chili peppers were chewed for 6 mm, and samples obtained applying the filter paper to the inferior surface of the turbinate for 20 sec. The ionic composition and sample weight of ASL for normal subjects and CF patients after stimulation of gland secretion were compared to paired values from baseline measurements (Fig. 3). After SMG stimulation, the volume of secretions increased (—3-fold to 3 5-45 mg) and the estimated osmolarity decreased (—30-40 mQsmJL). There were decreases in the concentration of Na^+, Cl-, and K^+ for both groups, but the anion gap tended to decrease in normals (12 to 6 mEq/L) and increase in CF (6 to 12 mEq/L). Taken together, these data suggest that a Cl^--rich hypotonic solution is secreted from normal and CF SMGs in response to cholinergic stimulus. The magnitude of the secretory response, and the watery nature of the secretions after chili peppers in both normal and CF subjects, suggest that nasal SMGs of CF patients can secrete an adequate, perhaps normal, volume of liquid in response to robust cholinergic stimulation, and the dominant anion of the hypotonic fluid is Cl^-. We speculate that the acinar region of CF SMG, like the acinus of the CF sweat gland, can secrete Cl^- via non-CFTR pathways, and the water-impermeable duct of the CF SMG has mechanisms to remove NaCl (unlike the CF sweat gland duct) from the isotonic primary acinar secretion. Any difference in the ion composition of stimulated nasal SMG secretions in CF must be subtle, but we will further study the different trends (p=0.06, difference between change in normal vs. CF) of the anion gap pattern between CF and normal by measuring the HCO_3^- and pH of SMG secretions. Another key measure of SMG secretions will be % dry wgts (water content), which may demonstrate a normal ratio of H_2O to solids as the secretion exits the cholinergic-stimulated SMG in CF.

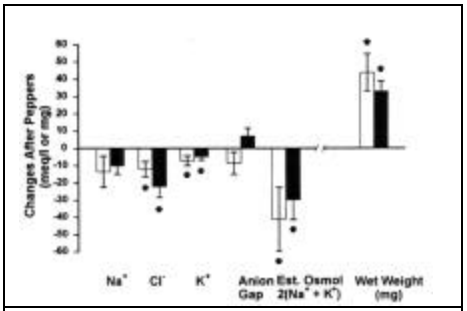

Figure 3. The change in volume (weight), ionic composition (Na^+, Cl^-, K^+) and estimated osmolarity of nasal ASL after stimulation of SMGs (as compared to baseline) in normals (n—8; open bars) and CF patients (n=8; solid bars). *p<005 - different from baseline.

lB. Bronchial surface liquid composition (filter papers)

We extended our measurement of the ionic composition of ASL to the bronchial surfaces using transbronchoscopic sampling of surface liquid with the filter paper technique. Studies were performed in normal subjects (mean age 26 yrs), CF patients who had no evidence of lower airway infection or inflammation (mean age 1.2 yrs), and chronic bronchitis patients with clinically stable disease (mean age 38 yrs) (Fig. 4). The ion composition of the bronchial ASL in normal subjects is different from the nasal ASL. The estimated osmolarity is hypotonic (~210 mEq/L). The concentration of Na^+ is *~15%* lower than nasal surface liquid and *~35%* lower than plasma, and the concentration of Cl^- in bronchial ASL is lower than either nasal ASL or plasma. The bronchial K^+ concentrations (like nasal ASL) are 3- to 4-fold greater than plasma. The ionic composition of ASL on the bronchial surface of uninfected CF and chronic bronchitis patients was similar to that in normal subjects. Importantly, the estimated osmolarity was also hypotonic in the CF and chronic bronchitis patients.

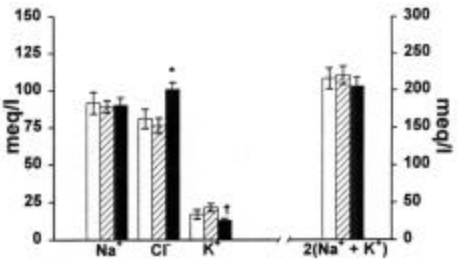

Figure 4. Ion composition (Na^+, Cl; K^+) and estimated osmolarity of ASL in proximal bronchi of normal subjects (n=l 1; open bars), CF patients (n=9; hatched bars), and patients with chronic bronchitis (n=9; solid bars).

Because nasal SMG secretions are hypotonic, we considered the possibility that SMG secretion was induced by the transbronchoscopic filter paper sampling technique, and contributed to the hypotonic ASL measured in proximal bronchi. To explore that possibility, we plotted the relationship between the estimated osmolarity [$2(Na^+ + K^+)$] and volume (weight) of ASL samples obtained from the normals, uninfected CF patients, and patients with chronic bronchitis (Fig. *5).* In each study cohort, the larger the sample weight on the filter paper (and by implication, the larger the rate of SMG secretion), the more hypotonic the ASL. In contrast, the smaller samples approached isotonicity. This suggests that SMG secretion is induced during bronchoscopy and sample collection, and we speculate that the ASL composition under baseline conditions is probably close to isotonic.

Bronchial and nasal ASL ion composition: ion-selective electrodes Na^+-selective electrodes to measure bronchial ASL: To test the accuracy of the filter paper sampling and analytic procedures, we measured the Na^+ concentration of bronchial ASL by a commercially-available Na^+-selective glass electrode (Microelectrodes, Inc.; Londonderry, NH). The concentration of Na^+ in bronchial ASL in 6 normal humans measured by Na^+-selective electrodes *in vivo* (—85 mEq/L) was similar to those measured with filter papers (Fig. 4). Also, the Na^+ concentrations measured by Na^+-selective electrodes in canine trachea (n=3) *in vivo* (~140 mEq/L) were comparable to values previously reported from filter papers (52). Bronchial tracings from a normal human, and Na^+ concentrations (90 and 93 mEq/L) in right mainstem (RMS) and bronchus intermedius (BI) ASL as derived from the standard curve, are shown in Fig. 6.

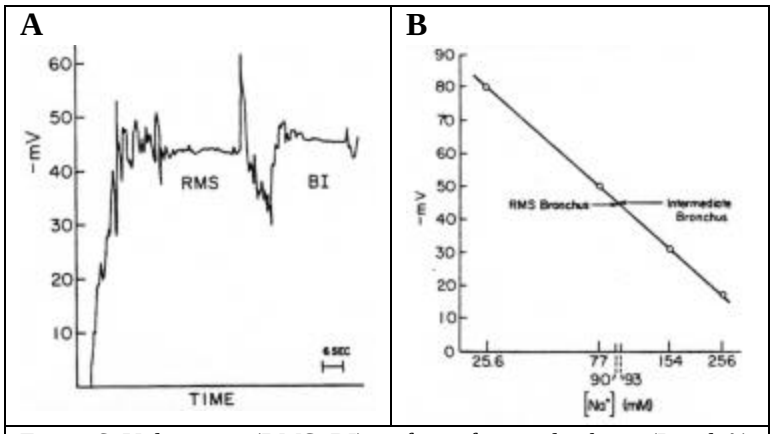

Figure 6. Voltage rec (RMS, BI) surface of normal subject (Panel A) and standard curve with notations (see arrows) of voltage recorded from bronchi, and derived Na^+ concentrations (Panel B).

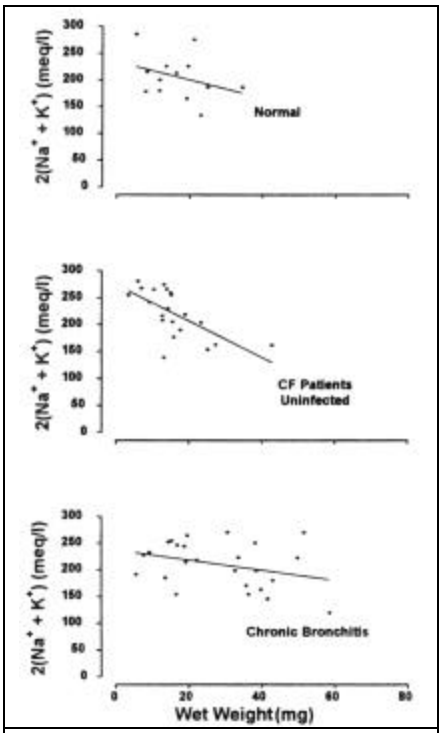

Figure *5.* Relationship between the estimated osmolarity and volume (weight) of surface liquid sampled from the proximal bronchi of normal subjects (n= 11), patients with cystic fibrosis (n=9), and chronic bronchitis (n=9).

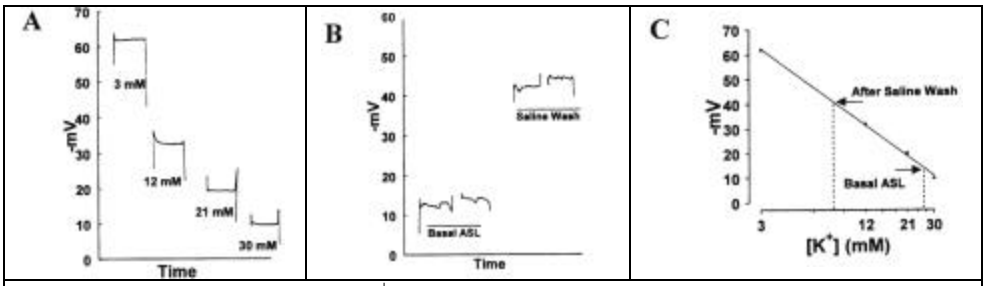

Figure7. Voltage recordings of K^+-electrode calibrated with known concentrations (3 to 30 mM) of K^+ (Panel A); tracings from nasal surface of normal subject under basal conditions, and after nasal spray with NaC1 *(150* mM) (Panel B); standard curve with notation (arrows) of voltages from basal ASL and after NaCl spray, and derived K^+ concentrations (dotted lines) (Panel C).

K^+-selective electrode to measure nasal ASL: We are testing small diameter (150 ìM) "double barrel" silver wire-based K^+-selective and reference (PD) electrodes provided by our collaborator (T. Johnson, Ph.D.). *In vitro* and *in vivo* studies of nasal ASL in a normal subject are shown (Fig. 7). The response time on the calibration standards is rapid (< 10 sec) and the voltage change is linear over a log-fold difference (3 to 30 mM) in K^+ concentration. *In vivo*, the voltage outputs when immersed in nasal ASL before and after saline spray are relatively stable, but voltages differ, measuring concentrations of K^+ (~26 and ~6 mEq/L; Panel C) that are similar to values from filter papers under similar conditions *(—25* and *5* mEq/L). After the *in vivo*

measurements, a repeat calibration curve reproduced the initial calibration. These data provide strong support for the validity of this approach with ISEs, and highlight the potential usefulness of these methods to measure ASL ion composition *in vivo.*

Specific Aim 2: Measure the mass of "mucus" and its state of hydration in large and small airways of normal subjects, CF patients, and disease control subjects.

B. Studies in the lower airways of normal subjects and CF and disease control patients (± infection): Bronchoscopic studies in CF children/infants

Children (< 3 yrs) Studied by BAL				
	Total	**Infected/Inflamed**	**Uninfectedflnflamed (>lO%PMN)**	**Uninfected/Uninflamed (lO%PMN)**
CF	31	16 (52%)	11 (35%)	4(13%)
Control	*45*	*26 (58%)*	*5 (11%)*	14(31%)

Previous studies from UNC have defined 3 subsets of airway status by BAL in CF infants: 1) infected and inflamed; 2) inflamed, but no infection; and 3) uninfected/uniflamed (62). Of the patients formally studied at UNC from 1995-97, the distribution of the subsets is shown in Table 1. As can be seen, 15 CF patients were uninfected, but only 4 patients (13% of CF pts. studied) had no infection or inflammation. The newly approved clinical research protocol in neonates with meconium ileus will clearly increase the number in the latter subset. The availability of BAL from a large number of CF and non-CF infants/children who are bronchoscoped for clinical indications, coupled to meconium ileus neonates, provides the opportunity for this project to rigorously test whether mucus stasis precedes infection, and whether inflammation can occur in response to non-infectious insults (?mucus stasis).

Quantitate "mucin" in BAL harvested by standardized techniques: Two assays have been developed to measure "mucin" in nasal ASL and BAL samples. Each of these assays (a PAS-slot blot assay and an ELISA assay] will yield units of "mucin equivalents", based on standard curves from purified CF mucin. The PAS assay takes advantage of the observation that PAS stains mucin-storing cells in the lung *(77-79).* The ELISA utilizes H6C5, a monoclonal antibody raised against mucins purified (80) from CF sputum. In standard curves for these assays (Fig. 8), the sensitivity limit for PAS is *25-5* 0 ng/ml, whereas the ELISA is more sensitive (~5-fold). Identical standard curves for the ELJSA are achieved with mucins purified from CF and asthmatic sputum and from JIBE cultures, as well as intact mucins and those reduced by exposure to 10 mM DTT (data not shown). To determine whether these assays are sufficiently sensitive to detect mucins in BAL fluids, we used "neat" BAL from the second Iavage sample (presumably containing less mucus than the first lavage sample) from subjects without lung disease and patients with mild stable chronic bronchitis (normal FEy_1 samples from separate study). In the PAS assay, BAL sample volumes were adjusted to yield blots in the linear range, representing BAL "mucin" concentrations of 0.1 to 6. 1 8 ìg/ml. As can be seen in the summary data (Fig. 9), we can easily detect mucin equivalents in BAL fluid from lavage samples. To make a similar determination for CF infants, the mucus in a 3 ml BAL sample was isolated (70 ìm nylon mesh cell strainer; Becton Dickinson) and solubilized with 300 ìl of 10 mM DTT. A 10 p.1 volume of this material yielded48óngmucin equivalents (BAL fluid concentration = 4.9 ìg/ml). We conclude that sufficient PASpositive material exists in BAL fluid samples to measure by this assay, and we anticipate that there will be adequate material for detection following isolation of high molecular weight glycoconjugates (HMWG) by hyaluronidase digestion and CL-4B chromatography.

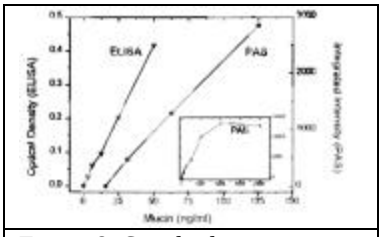

Figure 8. Standard curves, derived by H6C5 ELISA and PAS slot blot, with purified human mucin. The inset shows a PAS assay result over its full range.

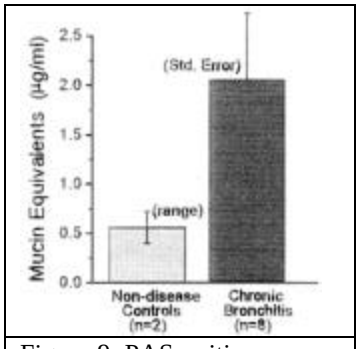

Figure 9. PASpositive glycoconjugates in BAL. Data are expressed relative to a purified human mucin standard.

Cellular and macromolecular composition of "plugs": We have developed techniques (see Methods; Spec. Aim 2B) for rapid transfer of mucus plugs from saline lavage fluid into inert perfluorocarbon (FC-72 A Fluorinert, 3M) followed by processing for frozen or TEM sections. Figure 10 shows frozen sections of a mucus plug from an 1 8-mo.- old CF patient with 40K cfu/ml oral flora in the BAL (Panel A). a 28-mo.-old CF patient with 20K cfu/ml oral flora and 60K cfu/ml *Xanthomonas* B *maltaphilia* (Panel B), and a 9-yr-old asthmatic patient with 10K cfu/ml oral flora (Panel C). The mean diameter of 7 CF mucus plugs (measured on frozen sections) was 1.30 mm (0.57 mm S.D.) and for 2 non-CF plugs was 1.58 mm. Hematoxylin and eosin (L1&E) staining shows that the mucus plugs are not homogeneous. The asthmatic plug section (Panel C) has more cells and eosinophilic material than the CF plugs. All three plugs have PAS reactive material consistent with mucin as shown on the PAS stain and all have DNA (stained with TO-PRO-1 iodide; Molecular Probes). The uninfected CF patient (Panel A) has the least DNA, most likely reflecting fewer PMNs.

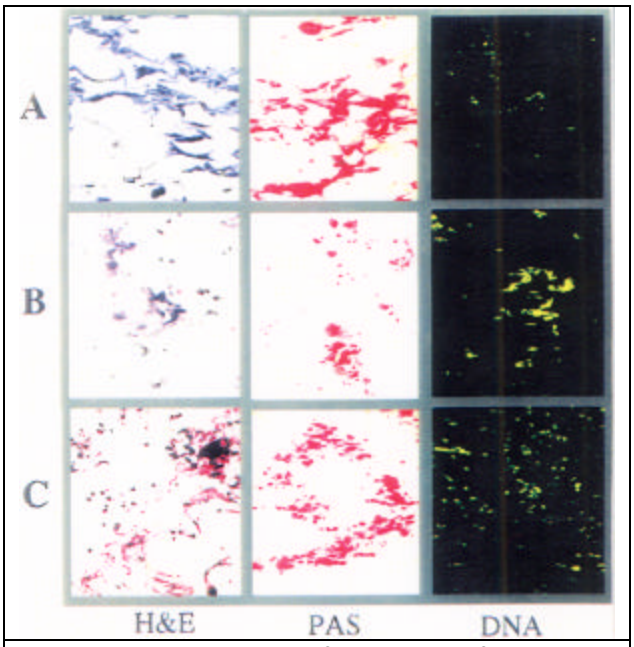

Figure 10. Frozen sections of mucus plugs from patients with CF (A and B) and asthma (C) (see text for comments).

d. EXPERIMENTAL DESIGN AND METHODS

This clinical-based project focuses on characterization of ASL of normal subjects, CF patients with a range of disease, and several pertinent disease-control groups using different techniques. To assist the reviewer, the rationale, protocol, and data analysis/limitations are described under each Specific Aim.

Specific Aim 1: Measure ionic composition of ASL in large and small airways of normal subjects, CF patients, and disease controls, and measure the relative contributions of surface epithelium and submucosal glands (SMGs) to the balance of airway surface liquid (ASL) ionic composition.

A. Nasal surface liquid composition and epithelial function *in vivo*

1. Rationale: Measurement of ASL ion composition will distinguish between isotonic volume absorption versus hypotonic ASL and provide information about SMGs in early pathogenesis of CF lung disease. The nose is accessible for repetitive measurements, reflects the complexity of proximal bronchi (surface and SMG epithelia), and has served as a useful model of lower airway ion transport function. Initial studies will emphasize the filter paper technique, supplemented by "first generation" ion-selective electrodes (ISEs) that are now available for *in vivo* human application. The ISEs will become increasingly useful as multi-polar (more than one ion measured simultaneously) versions are developed. The nose is also a site where we will use volume markers to make estimates of the relative rate of amiloride-sensitive volume absorption *in vivo* in CF versus normals and disease controls, and test the linkage between the rate of Na^+ transport to volume flow. These studies will be complemented by studies of drug-induced modulation of ion concentrations. Based on the isotonic volume hypothesis, we predict that a Na^+ channel blocker (amiloride) will lower ASL [K^+], reflecting a reduced driving force for K^+ secretion across the apical membrane and passive redistribution through the paracellular pathway in response to the transepithelial PD (reduced by amiloride), and an increase in [Na^+], reflecting inhibition of absorption. However, we predict that ASL will remain isotonic, with only the relative distribution of ions changing. Comparison of these responses in CF vs. normals will be informative. The effects of â-agonists or UTP on electrolyte composition may be modest, e.g., perhaps an increase in [Cl^-]. These protocols will also be sensitive to outcomes predicted by the hypotonic ASL hypothesis. For example, in normals one would predict that ASL [Na^+] and [Cl^-] will be raised by amiloride, reflecting the decrease in absorption of NaCl from ASL. Conversely, it is predicted that in CF, UTP may decrease [NaCl] in ASL by activating an alternative Cl^- channel, permitting transcellular absorption of NaCl.

There are several key disease control groups. Patients with PHA have mutations in the epithelial Na^+

channel and defective Na^+ absorption in multiple organs (68-70) and bioelectric properties in the nose consistent with no Na^+ transport (data not shown). The failure to absorb Na^+ from airways predicts isotonic ASL in PHA patients, but the pattern of ion concentration would mimic the predicted effect of amiloride (see above). Sjögren's syndrome is pertinent, because SMGs are ablated by lymphocytic immune mechanisms (71,72). If Sjögren's patients do not secrete from SMGs in response to cholinergic stimulation, the absence of typical CF lung disease may argue against the role of SMG dysfunction in CF airways pathogenesis. Finally, patients with primary ciliary dyskinesia (PCD) are pertinent because they have nasal inflammation and poor mucus clearance, and they have no genetic abnormality of epithelial ion (or water) transport (81,82).

2. Study Subjects/Methods:

a) Study subjects: We will study normal subjects (age 18-40 yrs) with no history of chronic rhinitis, and no acute nasal symptoms (2 wks). CF patients must fulfill standard diagnostic criteria, take no chronic nasal medication, and be without acute nasal symptoms (2 wks). Patients with systemic PHA must fulfill criteria of a generalized defect in Na^+ transport (i.e., elevated sweat and salivary NaCl and severe renal salt wasting). Patients with Sjögren's syndrome must fulfill standard diagnostic criteria, supported by serologies (71,72). PCD patients must have compatible clinical disease and a defined abnormality of ciliary ultrastructure (82). The study protocols will use 8-10 study subjects per cohort; study groups are listed by protocol (see below).

b) General methods

1. Filter papers are cut from Whatman 541 ashless paper (4 cm in length and *4-5* mm in width). After washing in double-distilled water, and drying overnight, the papers are folded in half width-wise and weighed just before sampling. To sample nasal ASL, the end of the filter paper is grasped in a hemostat and positioned under direct vision as a "V" laterally under the inferior turbinate for 20 sec. Serial weights are recorded (Cahn microbalance) over 90 sec with a regression line extrapolated to time 0 for the initial weight. Electrolytes are eluted (see Prelim. Data) and measured in the Analytic Core (J.T. Gatzy).

2. Ion-selective electrodes (ISEs): Initial studies with ISEs involved glass electrodes. Recently, we have been working with silver wire-based electrodes provided by T. Johnson, Ph.D. of the UNC Dept. of Bioengineering for *in vivo* measurements of ions (see letter of collaboration) (83). The cation-selective electrodes are constructed from Teflon-coated silver wire *(—150* p.M O.D.) with a semi-permeant coating selective for the pertinent ion. The electrodes are interfaced to a high-impedance voltmeter (84), values recorded, and ion concentration calculated, based on standard curves of voltage output over a log-fold change in concentration for each measured ion (37°C). Pilot experiments with the K^+-selective electrode indicate a response time <10 sec and a linear voltage change of 55 mV per log change in K^+ concentration. Similar data have been reported for the H^+ and Na^+-selective electrodes (T. Johnson). For data to be valid, reproducible standard curves must be obtained before and after *in vivo* measurements. For the experimental measurement, the ion-selective and reference electrodes are placed in a double-lumen polyethylene catheter (2 mm OD) and placed under direct vision under the inferior turbinate, until stable recordings of at least 10-15 seconds are achieved.

3. Volume marker to measure relative rates of ion-transport-linked volume flux in CF as compared to normal and disease controls, and relative water permeability: We will use an impermeant volume marker to estimate the relative rate of volume (and water) movement. Initial studies will employ ^{99}Tc-labeled albumin or DTPA, which we are currently using in other studies. To lessen the use of isotopes, we will pursue alternative strategies, i.e., fluorescent-labeled dextran. We will spray the nasal surface with a test solution containing the marker. Serially over time, we will sample nasal ASL at different sites with the filter paper, measure changes in marker concentration, and estimate relative rates of volume flux as outlined below.

3. Protocols:

a) Baseline ASL composition

1. Filter paper sampling: After occluding the nostrils for 10 minutes, nasal ASL will be obtained and samples analyzed for (Na^+, K^+, Cl^-, and HCO_3^-). We will measure divalent ions that may be relevant to mucus gel formation or antimicrobial activity (Ca^{2+}, Mg^{2+}, and Fe^{2+}).

2. Ion-selective electrodes (ISEs): After occluding the nostrils for 10 min, ISEs will measure

the concentration of Na^+ , K^+, H^+ (pH). The ISEs will be positioned on the inferior surface of the inferior turbinate, which is the site of our previous functional studies of ion transport by nasal PD (84) and the locus of sampling ASL with filter papers (see Prelim. Data). Patients will breathe through the mouth, and pilot experiments suggest that compositions are stable if ions can be measured in 90 sec.

b) Surface **epithelial modification of ASL: We will** use ipratropium (Atrovent) nasal spray to inhibit SMG secretion (74,75). Four sprays (168 ìg) of ipratropium (vehicle is 150 mM NaCl) will be applied to the surface of the inferior turbinate and floor of each nasal cavity under direct vision. This dose of ipratropium is effective at inhibiting methacholine-induced (cholinergic) nasal SMG secretions, and lasts at least an hour. Twenty to 30 minutes after ipratropium pretreatment, nasal ASL will be collected by filter paper, and/or ion concentrations measured by ISEs, under several conditions:

1. Basal ASL ion composition: After occluding nostrils for 10 mm, we will collect ASL by filter papers from normal, CF, and disease controls, and analyze for Na^+, K^+, Cl^-, HCO_3^-, Ca^{2+}, Mg^{2+}, Fe^{2+}. The pH, Na^+, and K^+ will be measured by ISEs.

2. Surface epithelial modification of test solutions of defined ion composition: To test elative permeability of the surface epithelium to Cl versus HCO; (see Prelim. Data), the nasal mucosa will be spray-coated with saline (150 mM NaCl) or Ringer containing-HCO_3^- *(*25 mEq/L). The nasal surface will be sampled immediately to define the starting conditions of ion composition. After nasal occlusion for 10 and 20 minutes, nasal ASL will be resampled to measure the change in ionic composition reflecting surface epithelial ion transport/permeation. ISEs will be used to measure pH.

3. Effects of pharmacologic modulators of ion transport on ASL ion composition: We will co-administer ipratropium (block SMG) with inhibitors of active Na^+ absorption (amiloride, 10^{-3} M), activators of Cl^-secretion (terbutaline 10^{-4} M, UTP 10^{-3} M), and combinations of amiloride ± terbutaline or UTP. After 20 mm, the nose will be occluded and samples obtained for electrolyte analyses 10' and 20' later.

4. Estimates of relative volume flux associated with active ion transport: We will study CF and compare rates to normals and disease controls. We will measure the rate of change of the concentration of volume marker after spraying the nasal mucosa with isotonic NaCl solutions and sampling ASL with filter paper over time. Estimates of volume flow associated with active Na^+ transport (3-8 pl/cm^2 hr) (2-4,14) suggest that sampling after 20 to 30 minutes should sense changes in the concentration of the volume marker. Regulation of volume flow will be tested with amiloride, 1 0~ M (or replacement of Na^+ with choline) in the spray solution, which should block Na^+ (and volume) absorption. Conversely, amiloride + terbutaline (l0~ M) or UTP (10~ M) in the spray should lead to Cl^- (and volume) secretion, which should dilute the volume marker. Finally, putting terbutaline or UTP alone in the spray will test whether activation of CFTR or CPa induces Cl^- secretion (dilution of volume marker) or volume absorption. Lidocaine (4%), which blocks all ion transport, will be a control for non-ion transport-mediated changes in volume marker concentrations.

5. Estimates of relative rate of water permeability: We will estimate water permeability by measuring the rate of change in the concentration of the impermeant volume marker in hypotonic (NaCl 25 or 50 mM) electrolyte solution and isotonic solution without electrolyte (sucrose/mannitol) that are sprayed onto nasal surface. Sequential samples will be obtained by filter paper (nostrils will be occluded otherwise) and the concentration of marker and ions plotted per time. Pilot experiments using hypotonic saline *(50* mM NaCl) suggest that excess water is absorbed over —6-8 minutes (i.e., nasal ASL becomes isotonic after ~6 minutes). Therefore, sequential samples will be taken at intervals of 1-2 minutes.

c) Determine SMG secretory function: We will study normal, CF, and disease controls. After occluding the nostrils for 10 minutes, subjects will chew chili peppers for 6 minutes to induce SMG secretion (4 ***1,76,85),*** and samples will be obtained by the filter papers. Excess secretions will be blown into a specimen cup, and stored (20^0C) under water-saturated mineral oil. Electrolyte concentrations will be measured (Na^+, Cl^-, K^+, HCO_3^-). We will use ISEs to measure pH, Na^+, and K^+ of nasal ASL *in vivo*. Divalent cations that may be important in hydration of the mucin gel or airway host-defense will be measured: Ca^{2+} (as ratio to Na^+ relevant to hydration of gel), Mg~ (defensins); Fe^{2+} (bacterial growth).

4. Data Analysis/Limitations: In normals, we anticipate that the baseline ASL will be isotonic, but with different concentrations of Na^+ and K^+ as compared to plasma. If true, this would be a seminal observation

relative to our central hypothesis. The analysis of divalent ions will provide a more complete picture of normal ASL. We predict that the ASL will remain isotonic on surface epithelium (after inhibition of SMG with ipratropium), but the concentrations of individual ions may vary. Because of the possible importance of ASL HCO_3^- (and pH), we will test solutions of varying Cl^--HCO_3^- ratios. These experiments can be difficult to interpret because differences in [HCO_3^-] or pH can reflect differences in Cl^- vs. HCO_3^- permeation through the paracellular path or cellular Cl^--HCO_3^- exchange (see Project I), or possible HCO_3^- secretion via CFTR (86,87). The ultimate interpretation will rest on joint studies with Project I *in vitro* and local collaborators (A. Paradiso, Ph.D.) who are studying CF and normal airway epithelia *in vitro*, and have not yet found evidence for an apical H^+-ATPase or Na^+/H^+ exchange (88). For our experiments, percent change of the administered concentration of Cl^- and HCO_3^- will be used to determine the relative permeabilities. The effect of pharmacologic regulators will be analyzed next. We predict that amiloride will not change the tonicity of ASL but will lower the [K^+] and raise [Na^+], and this pattern may mimic PHA. Terbutaline and UTP also will not change tonicity but may raise [Cl^-]. Finally, two key experiments will follow up on our initial characterization of ASL as hypo- or isotonic. First, if ASL is isotonic, we speculate that plots of ASL ion composition (and volume marker) vs. time following application of hypotonic liquids will rapidly change (approach isotonicity and concentrate marker), consistent with a physiologically significant water permeability. Second, if we observe isotonic ASL, we predict isotonic volume flow. Amiloride-blockable concentration of our volume marker and amiloride/UTP dilution of our volume marker would be consistent with this hypothesis. We anticipate that SMGs will secrete a hypotonic fluid in response to cholinergic stimulation (see Prelim. Data).

We will then proceed to analyses of ASL in CF patients. Based on Prelim. Data, we anticipate that ASL will be isotonic, with concentrations of Na^+, K^+, and Cl^- similar to normal subjects. An important extension of this characterization will be to divalent cations. We predict that the ASL on surface epithelia (after SMG block) will be isotonic, but the ion composition may differ in CF from normal if there is a different rate of Cl^- (versus other anion) permeation. For example, if the large difference in anion gap between normal and CF (26 versus 1 mEq/L, respectively; see Prelim. Data, Fig. 2) reflects differences in HCO_3^- concentration, the pH of CF ASL is predicted to be different. This will also be tested with ISEs (pH), and correlated with relative changes in Cl^- vs. HCO_3^- (see above). Next, it will be important to compare the pattern of electrolytes following drug (amiloride, terbutaline, UTP) in CF ASL to normals. The isotonic volume hypothesis predicts that amiloride effects on ASL composition will be similar to normals: decreased [K^+]; increased [Na^+]; persistence of isotonicity; whereas the hypotonic ASL hypothesis predicts little change in CF, but large changes in normals (see above). The effects of UTP alone may be instructive. The isotonic volume hypothesis predicts perhaps slight increases in [Cl^-] in both CF and normals. The hypotonic ASL hypothesis might predict little change in ion composition in normals and decreases in [Cl^-] in CF. Thus, the pharmacologic protocols will increase our capacity to distinguish between these two hypotheses and perhaps most importantly give us clues as to the consequences (good or bad) of pharmacologic modulators of ion transport for therapeutic uses. We will plot the relationship between composition vs. time for the applications of hypotonic fluids. A rapid (6-8') return to isotonicity would be consistent with a relatively water permeable epithelium, and these parameters will be compared to normals. Finally, we will analyze the rate of concentration of the volume marker, the possible effect of amiloride block of volume marker concentration, and the effect of UTP (or terbutaline) ± amiloride. Comparison of basal and amiloride blockable volume marker concentrations with normals will directly test our hypothesis that Na^+ transport-dependent volume absorption is increased in CF. Regarding SMGs, we anticipate secretion of a normal volume of hypotonic liquid in CF under robust cholinergic stimulation, and the predominant anion will be Cl^-, which implies no significant defect in the ability of CF SMGs (at least in nose) to secrete volume and water. However, the different pattern of change in anion gap (see Prelim. Data, Fig. 3) suggests that HCO_3^- (or another anion) may be secreted by CF SMGs, and measurement of HCO_3^- (and pH) will be important. Finally, it will be critical to compare disease controls and the effects of mutated ENaC function on ion composition of ASL; i.e., do patients with PHA, who exhibit airways disease early in life, but do not develop chronic bacterial infection, have iso- or hypotonic ASL and does the pattern of electrolytes mimic amiloride administration? Similarly, will patients with Sjögren's have any unexpected findings that may be consistent with the absence of lung disease, e.g., hypotonic ASL?

The limitations of these protocols largely relate to technical considerations, and the relevance of nose to lower airways. Most of the technical issues related to sampling small volumes and measuring ionic constituents from filter papers have been addressed (see Prelim. Data). We recognize the need for sensitive, rapid, and small ISEs to extend our capabilities, and we are optimistic that our current collaboration (see letter, T. Johnson, Ph.D.) will allow rapid application of ISEs to human airway surfaces *in vivo,* including lower airways (see below). We recognize that we cannot measure "water permeability" *in vivo* it is hard enough *in vitro* where one can control volume/surface area and mixing. However, our goal is to measure "biologically relevant" water permeability, i.e., we wish to know whether the epithelium can maintain an anisosmotic solution or not, and whether there are differences in CF. Our simple technique should answer these questions. Although the approach to estimating volume flow *in vivo* is in development, volume markers are used for these measures *in vitro* ([see Project I and (89-9 1)]. The major problems are the possible effects of repetitive sampling (we shall use separate sites), surface movement of the marker via ciliary action (which we hope to circumvent by spraying a large area), and the dilution of marker either via SMG (which we hope to completely block with ipratropium) and/or exudation due to filter paper damage. At a minimum, we should be able to determine whether CF surface epithelium has a 2-3 fold greater rate of volume flow (concentration of marker) than normals that is inhibited by amiloride. Regarding the nose as a model of proximal lower airways, epithelial ion transport functions are similar for these two regions. If parallel functions in nose and lower airways with more extensive studies of ASL ion composition show a similar parallelism in these parameters, the accessibility of the nose for complex experimental protocols provides the opportunity to study key physiologic events. We emphasize availability of at least 10 patients in each disease-control population (including PHA and PCD) who are already participating in other research protocols with us.

B. Studies of lower airway epithelial function

1. Rationale: The key measurement to distinguish between the two major hypotheses of salt and water metabolism by airway epithelia is the ion composition (and tonicity) of ASL. Our preliminary studies showed no difference in bronchial ASL tonicity or ionic composition between CF and normal subjects, but the bronchial ASL collected was hypotonic in both groups. We speculate that the hypotonic bronchial ASL is an artifact of collection-induced SMG secretion, and the bronchial ASL under baseline (unperturbed) conditions is isotonic. To test this speculation, we will take two approaches. First, we will measure ASL composition, after topical ipratropium to block SMG secretion, in proximal airways of normal adult subjects (and, ultimately, CF infants). These studies will initially be performed with filter papers, and subsequently with ISEs. The second approach involves the use of ISEs to measure ASL ion composition on distal airway surfaces, which contain few (or no) glands (1,92). Because the smaller airways are less influenced by SMG, i.e., 6-8k" generation bronchi in adults have *<5%* of the SMG volume per airway surface area compared to proximal airways (mainstem bronchus), the contribution of any reflex SMG secretion is likely to be small, and less confounding to measurement of ASL ion composition (92). Measurement of ASL in distal airways will have an added benefit in that it will provide information about ionic composition of ASL in airway regions in uninfected CF infants that is critical to test our hypothesis and our understanding of early pathogenesis. Finally, our Prelim. Data show that there is no anion gap in CF ASL on nasal surface epithelia (SMG secretion blocked by ipratropium), whereas there is a 25 mEq/L anion gap in normal subjects. These data suggest that CF, but not normal, surface epithelia absorb another anion (?HCO_3^-) preferentially to Cl^- [or secrete more H^+, which seems unlikely (88)]. The possibility that HCO_3^- may be present in greater concentration in normal versus CF ASL will be tested by measurement of HCO; using filter papers, and pH by ISEs.

2. Study subjects and General methods:

a) Study subjects:

1) Adult: We will study normal subjects (age 18-35 yrs; —10 for each protocol) with no history of airways disease, no acute respiratory symptoms for at least 2 weeks, and no chronic medication use. We currently have IRB permission to perform transbronchoscopic studies with filter papers and ISEs.

2) Pediatric: Children and infants aged 3 years or younger who are undergoing clinically indicated bronchoscopy and BAL at UNC Children's Hospital will be recruited for participation in the studies. Based on recent clinical data, we anticipate about 120 infants per year would be eligible for the study (~40 with

CF). The remainder will provide a "normal" control population (structural airway abnormality) and a "disease" control population (aspiration syndromes, asthma and recurrent pneumonia). We currently have IRB permission to perform bronchoscopic studies with filter papers.

b) General methods

1) Filter paper technique: For pediatric studies, three filter paper (Whatman 541) strips (each 1cm x 0.15 cm) are held by a biopsy forceps in the distal lumen of the suction channel of a 3.8 mm flexible bronchoscope. The distal tip of the channel lumen is sealed by a plug of polyethylene glycol to prevent contamination of the filter papers with upper airway secretions. The bronchoscope is advanced into a segmental bronchus and the forceps advanced to expel the plug and advance the filter papers, which are exposed to the bronchial surface for 20 seconds, then withdrawn into the suction channel. The bronchoscope is then withdrawn from the patient with the filter paper in the channel. Adult studies will be performed as described (93,94). Briefly, sterile, pretared filter papers *(*n=5; 1.5 x 15 mm) clasped in a biopsy forceps are introduced transbronchoscopically into the airway lumen with a protective sheath and polyethylene plug in the distal end. The forceps with filter papers are advanced, touched to the airway surface for 20 sec, withdrawn into the sheath, and removed from the bronchoscope (see Spec. Aim lA). The filter papers will immediately be weighed on a microbalance to determine wet weight. One set of filter papers will be used for wet/dry weights, one each for ionic composition and osmolality (Analytic Core). The methodologies for preparation of filter paper, extrapolation of wet and dry weights, and ion/osmolality analyses are described in detail for studies in Specific Aim 1A.

2) Ion selective electrodes (ISE; transbronchoscopic): Cation-selective electrodes are constructed from silver wire *(*150 ìM diam.) with semi-permeant covering that is selective for the appropriate ions (83), and the reference electrode is of similar dimension (150 ìM diam.). Both electrodes have no required "depth of immersion". ISEs will be selected that reproduce standard curves over a log-fold change in ion concentration at 370 *in vitro,* and we will record voltage outputs on a high-impedance voltmeter, safe for use in humans (84). We have permission to study ASL ionic activity with ion-selective electrodes (ISEs) in adult subjects, and the current ISEs are easily packaged in a polyethylene protective sheath (2 mm O.D.) that can be passed through the channel of standard bronchoscope. We will also be able to use a smaller bronchoscope (Olympus prototype, outside diameter 2.7 mm) in adults to extend measurements to distal airways, pending the construction of a protective sheath that will pass through the bronchoscope channel (0.9 mm diam). We plan to extend these studies to CF and control infants and children once the technique has been refined and institutional approval has been obtained (see below).

3. Protocols:

a) Proximal airways

1) Adults: We will initially use filter papers, and subsequently ISEs, to measure ion composition of bronchial (mainstem, lobar) ASL in normal adults after ipratropium pretreatment to block reflex SMG secretion. For the experimental measurement, the electrodes will be passed through the bronchoscopic channel in a protective sheath and extended to lightly touch the airway surface under transbronchoscopic vision until stable recordings (10-15 sec) are achieved at 3-5 sites in each bronchial region. The initial approach will involve ipratropium (168 ìg) sprayed transbronchoscopically onto the surface of one mainstem bronchus. This dose should be sufficient for inhibition of cholinergic-stimulated SMG, based on inhibition of nasal SMG secretion. After 30 minutes, ion composition of mainstem bronchial ASL will be measured using filter papers, and subsequently ISEs. The opposite mainstem bronchus (i.e., no ipratropium) will be tested as a control, i.e., we expect to recover hypotonic ASL. If the topical spray dose inhibits SMG secretion, i.e., we measure isotonic ASL on the treated side and hypotonic ASL on the placebo side, we will initiate protocols to test aerosolized ipratropium (168 ìg), which may be sufficient for at least partial inhibition of SMGs, based on estimates from the effect of aerosolized atropine to block cholinergic-stimulation of SMGs (95,96).

2) Pediatrics: Filter paper studies will involve children under 3 years of age undergoing clinically indicated bronchoscopy. Informed parental consent will be obtained. Sedation/analgesia will be provided by conscious sedation (midazolam and fentanyl) or general anesthesia (propofol). Patients will placed in the reverse-Trendelenberg position to prevent upper airway secretions and local anesthetic from entering the

trachea. Lidocaine (2%) will be applied to the nasal mucosa and onto the vocal cords. In some instances it may be clinically indicated to intubate the patient for the bronchoscopy. Studies will initially be conducted without ipratroprium, but we plan to proceed to pretreatment with topical spray (or inhaled) ipratropium, if successful in adults. The bronchoscope containing the filter papers will be then be positioned in the right mainstem bronchus, applied to the surface epithelium for 20 seconds, withdrawn into the suction channel, and bronchoscope removed. While an assistant processes the filter papers, the investigator will re-pass the bronchoscope into the lower airway for BAL sampling.

b) Distal airways, using ISEs. These studies will initially be performed in adults. We will measure the ion concentration in ASL from proximal sites (mainstem bronchus) to the most distal site possible using the pediatric (2.7 mm OD) bronchoscope. This should allow measurement of ASL in $_{6th}$ generation bronchi under direct vision, and the ISEs can be extended (2-3 cm) for measurements of ASL in more distal generations of bronchi (8-1 $_{0th}$) We anticipate performing measurements with unipolar ISEs that are currently available (K^+; H^+) and nearing availability (Na^+). The measurement of Na^+ and K^+ will give an estimate of osmolarity [$2(Na^+ + K^+)$]. We envision that the current development of multi-polar (2 or 3) ISEs will allow us to measure several ions simultaneously by year 2 (or 3) of this project.

4. **Data Analysis/Limitations**

a) Proximal airways: These studies will build on earlier work that measured ASL in normal adult volunteers, adult disease control and uninfected infants with CF. The use of iprotroprium in normal (and CF) subjects will be a critical part of the evaluation of baseline "unperturbed" ASL. We will compare values from filter paper and ISE ± ipratropium, and predict finding isotonic ASL in ipratropium pretreated subjects as evidence that the basal (unperturbed) composition of ASL is isotonic. Although the ASL may be isotonic in CF and normal bronchi, the pattern of electrolytes may differ between the 2 groups. For example, the pH and concentration of HCO_3^- in ASL will be measured directly for the first time to test whether HCO_3^- is responsible for the anion gap in nasal ASL of normal subjects (and absent in CF) after pretreatment with ipratropium, and whether HCO_3^- is relatively absent on CF (as compared to normal) bronchial surfaces. Measurement of osmolality by freezing point depression will be compared to the ion composition analysis as our independent check vis-à-vis whether ASL contains significant amounts of non-ionic osmolytes. The % dry wgts (and water content) of samples from normals and disease controls will be critical for comparison of % dry wgts of plugs from distal airways of uninfected CF neonates (see Spec. Aim 2B).

b) Distal airways: The use of ISE, and the development of more sophisticated versions (simultaneous multi-ion measurements) of ISE, is one of the most important innovations of this project. The use of current unipolar (K^+, H^+, Na^+) ISEs will provide an important approach to measure ion composition (and estimate tonicity, i.e., [$2(Na^+ + K^+)$] in distal airways. It is likely that we can measure ASL in 6-10th generation bronchi in adults, and since there are few glands, we predict isotonic ASL. We cannot use ISEs in children now, but we will extend their use to study distal airway ASL when ISEs are refined. One of the key elements of initial pathogenesis of CF is ion composition and its role in lung disease. When we study CF infants, we expect isotonic ASL, but the pattern of electrolytes in distal airways may differ from normal, since there are few glands, and CF airways may absorb HCO; in preference to Cl.

The principal limitations of filter paper studies are: Sampling is limited to the large airways, and mechanostimulation of SMG may release mucus and hypotonic liquid into the ASL. Systemic atropine in doses (0.6 mg) used during bronchoscopy does not block reflex-induced SMG in the bronchi, but topical ipratropium is adequate in the nose. We propose to use a transbronchoscopic spray device to deliver a dose equivalent to the nasal dose to a similar surface area in the mainstem bronchus. If successful, this approach will provide a clear answer, and may lead to testing ipratropium dosing by the aerosolized route. Ipratropium is very safe when administered by aerosol; the limit for adverse side-effects is a total dose greater than 1 mg (97,98), and a greater than usual inhaled dose is feasible. The use of microelectrodes attached to fine polyethylene tubing will allow study of more distal airways. These electrodes appear to have fast response time, are not fragile (they were developed for direct cardiac puncture), and have a shallow depth of immersion. The major difficulty may well be to localize "how distal" our measures are. We will use a combination of visual inspection and anatomic location.

Specific Aim 2: Measure the mass of "mucus", and its state of hydration in large and small airways of normal subjects, CF patients, and disease control subjects.

A. Studies of nasal mucosa

1. Rationale: Testing the overall hypothesis of this proposal (i.e., excessive isotonic volume absorption impairs mucus hydration on airway surfaces in CF) requires, in part, measurement of water content of mucus on airway surfaces. The nasal epithelium is a useful site to test this concept: 1) the composition of nasal ASL is not confounded by addition of ASL from other regions (as may occur in the bronchi with MCC); 2) nasal ASL can be sampled without inducing overt SMG secretion; and 3) SMG secretion can be blocked with ipratropium to study the regulation of mucus hydration by surface epithelia. This Aim also presents the opportunity to directly test the role of SMGs in the generation of abnormal ASL in CF. We will perform studies in the nose to test the concept of abnormal hydration of mucus on CF airway surfaces, and compare SMG function in normal, CF, and disease controls. We will measure the "mucin" and water content of nasal ASL, expressing these data as % solids of ASL, under several conditions: 1) basal; 2) after inhibition of SMGs with ipratropium; and 3) after stimulation of SMG secretion by hot chili peppers or topical methacholine. We will also measure ions that may be important in the hydration of the mucus gel (Ca^{2+}/Na^{+}; HCO_3^{-}/pH).

2. Study Subjects/General Methods:

a) Study Subjects: We will study normal subjects, CF patients, and disease-controls, i.e., patients with PCD and Sjögren's syndrome. Inclusion and exclusion criteria are as defined under Specific Aim 1A. We anticipate 8-10 subjects will be sufficient for each protocol.

b) General Methods:

1. Sampling ASL with filter papers to measure water content: Filter paper pledgets (Whatman 541; 4 cm x *5* mm) will be used for collection of ASL. To measure the percent dry weight of ASL samples (and water content), it is necessary to measure a desiccated dry weight of the filter paper, determined from immediate and sequential weighing (90 sec) of filter papers after overnight drying in an oven (100°C), and extrapolating back to time 0 for the initial weight. The filter papers are then allowed to equilibrate to ambient conditions, and a repeat weight is obtained before sampling. After sampling, immediate weighing will measure the wet weight of the sample, followed by a weight after drying in an oven (l00°C) to a steady-state desiccated weight; the difference between the wet and desiccated wgt is the dry weight of the sample. The % solids is then calculated as the dry wgt/wet wgt x 100. Pilot experiments indicate that it takes 48-72 hrs for complete desiccation of ASL to occur. We will also have macrosamples *(*0.5 to 1 ml) of SMG secretions induced by chili peppers or mechacholine (41), which are placed under water-saturated mineral oil and frozen.

2. Mucin assays: Samples will be eluted in 10 mM DTT, and "total mucin" will be measured by two approaches:

a. PAS reactive material quantitated by slot blot assay: 300 ìl samples are applied to nitrocellulose using a slot blot vacuum manifold. The membrane is air-dried and then incubated 30 mm with 1% periodic acid/3% acetic acid. Following a 2X wash with 0.1% $Na_2S_2O_5$ in 1 mM HC1, it is incubated 15 mm in Schiffs reagent, washed again, and dried. The blot is digitized and analyzed using *MetaMorph* image software to determine area under each peak. Samples of purified human mucin are included on each blot for a standard curve (see Prelim. Data).

b. Mucin (CHO) antigen quantitation by H6C5 ELISA: 100 ìl samples are bound to 96-well high-binding microtiter plates (Costar #3590) overnight at 4°C, or for 2 hours at 37°C. The plates are washed with PBS containing *0.05%* Tween 20 and 0.02% Thimerosol, and incubated with *1-5* j~g/ml H6C5 for 1 hour at 37°C. The plates are washed, similarly incubated with HRP-conjugated 20 Ab, and developed with an incubation in 0.04% wt/vol of the substrate, O-phenylenediamine. The reaction is stopped, and the optical density at 490 nm determined in a microtiter plate reader. Samples of purified human mucin are included on each plate for a standard curve (see Prelim. Data).

3. Lactoferrin/lysozyme assays: Lysozyme activity will be measured by following the decrease in OD405 of suspensions of *Micrococcus lysodeikticus (M lys.)* in 96-well microtiter plates relative to a standard.. For lactoferrin, a commercially available Elisa kit (R&D Systems, Minneapolis, MN) with a sensitivity for lactoferrin of 1 ng/ml will be used.

4. Na^+ and HCO_3^- analysis will be performed by the Analytic Core.

3. **Protocols:**

We will measure the mass of "mucin" collected and state of hydration under three conditions:

a) Baseline: After occluding the nostrils for 5, 10, and 20 mm, nasal ASL will be sampled by filter papers for (1) wet weight, desiccated dry weight, and electrolyte; and (2) mucin mass.

b) Effect of unopposed surface epithelial ion transport on mucus hydration: Ipratropium (nasal spray, 168 ìg) will be used to block SMG (details under Specific Aim lA). After 30 mm, the nostrils will be occluded for 5, 10, and 20 mm, and samples obtained by filter paper for measure of water content (% solids), electrolytes, and quantitation of mucin. Following these studies, we will spray a combination of ipratropium (168 ìg) and amiloride (l0~ M) ± UTP (l0~ M), or UTP alone, onto the nasal surface, wait 20', plug the nose for 10 mm, and collect samples for analysis of water and ion content.

c) Mucin and water in SMG secretions: After occluding the nostrils for 10 mm, subjects will chew chili peppers for 6 mm to induce SMG secretion, and samples will be obtained (by filter paper and nasal expectoration into water-saturated mineral oil) for measures of percent dry weight (water content), quantitation of mucin mass, and quantitation of lysozyme and lactoferrin.

4. Data analysis/Limitations: We will first calculate from wet and dry wgts the % solids as an index of the hydration of nasal mucus under basal circumstances for normal, CF, and pertinent disease controls. We will next estimate the mass of mucus by quantitating "mucin" and multiplying by volume of nasal ASL sampled. We predict, based on the isotonic volume hypothesis, that the percent solids of ASL under basal conditions will be increased in CF as compared to normal and disease controls. It is possible that the absolute mass of mucin may be increased in CF and disease controls (i.e., increased dry weight of sample, and increased amount of mucin by assays), which may reflect non-specific stimulation of mucus secretion from SMG and goblet cells by chronic inflammation. In that circumstance, we predict that the water content will still be decreased in the CF samples compared to the disease controls. The dehydrated state of CF mucus may also be accompanied by an increased ratio of Ca^{2+}/ Na^+ , reflecting inadequate ion-exchange and volume hydration of the mucus gel. Our assays will be sensitive to the predictions of the hypotonic ASL hypothesis, i.e., that the hydration of mucus will not be different in CF.

In equivalent analyses of surface epithelium (SMG blocked), we expect to see greater differences, i.e., more dehydrated mucus in CF reflecting liquid (volume) absorption without SMG secretion. An especially important analysis will be the effect of amiloride ± UTP on % solids. The prediction of the isotonic volume hypothesis is that following amiloride, water content will increase modestly in normals (i.e., % solids will modestly decrease), whereas there will be larger increases in water content (i.e., large decreases in % solids) post-amiloride in CF. Further, we predict that UTP with amiloride will have a selectively larger effect on water content in CF compared to normals. A key comparison will be the effect of UTP alone in CF versus normals on water content, i.e., does UTP alone trigger volume secretion *(15)* or absorption. The hypotonic ASL hypothesis predicts no (or trivial) changes in water content by these maneuvers.

With respect to SMG secretion, we predict that water content and mass of mucins will be similar in CF versus normal and disease controls, although the pH of basal ASL and SMG secretions may be altered in CF (see Specific Aim 1 A.) We will also compare lysozyme and lactoferrin as markers of SMG serous cell secretion, in CF versus normal and disease controls, to address the role of SMGs in the pathophysiology of CF lung disease.

The limitations of these nasal studies largely reflect the accuracy of measurement of water content and quantitation of "mucins", and the adequacy of the nose as a model for mucus secretion and hydration on lower airway surfaces. Pilot studies measuring the dry weight of samples of human ASL (10-20 mg, wet weight) suggest that we can reproducibly determine small percent dry weights (down to 3%), and rigorous attention to detail argues that we can detect a small change in percent dry weight, e.g., a change from 4 to 8% of dry weight of sample, which would be a change in the water content from 96% to 92%. We recognize the limitation of our "mucin" assay, but the relatively small size of the filter paper samples limits the ability to purify' mucins. We adapted a simple assay of PAS reactive material, which will be supplemented by an ELISA (antibody). The overall rationale for our strategy, and plans for development of other assays, is discussed in detail under Spec.

Aim 2B-l (see Data Analysis/Limitations). The nose has a complicated mucosa (see below) with a large ratio of SMG to superficial epithelium. For studies of drug modulations of mucus water content, we have elected to use ipratropium to block SMG function. Previous studies have detected little cholinergic regulation of superficial epithelial function (3) so we believe this strategy is valid. The effects of amiloride as noted above should persist throughout the measurement period. Quantitation of the effects of UTP are more complex. We are in the process of determining the duration of UTP action after nasal spray, but based on the duration of action via aerosol to the lung (40-80'), we believe the duration will be adequate for this study. UTP also may have effects on mucus secretion by goblet cells (99,100). However, we should be able to estimate a semi-quantitative effect by measures of mucin mass (an important issue in its own right) and we still should be able to ask the critical question, is the ASL after UTP more hydrated (% solids decreases). Regarding the nose as a model, adult CF patients have a chronic inflammation of the nasal mucosa, which may perturb the mucus secretory system. Inclusion of disease-controls will enable us to identify the effect of non-specific events. The nose is likely to be a good model of proximal conducting airways, (directly tested, Spec. Aim 2B), but it is unclear whether the nose will correlate with small airways, which do not have SMGs.

B. Studies of lower airways.

Overall rationale: These studies will directly test our hypothesis that CF lung disease is initiated by isotonic dehydration of mucus and its ineffective clearance from airways. We propose that the amount of mucus in CF airways is normal at birth, but accumulates over the first days to weeks of life, even in the absence of infection or inflammation. This retained mucus forms plugs of dehydrated mucin gel in distal airways and predisposes airways to infection, infiltration of PM7Ns and consequent hypersecretion by goblet cells and SMGs. After infection, the additional mucus contains PMNs, bacteria, DNA and F-actin that further increase the rigidity of the mucus. Conceivably, bacteria within mucus plugs may be "isolated" from antibacterial defenses and serve as continued stimuli for PMN infiltration. Our protocol is sensitive to outcomes predicted by the "hypotonic ASL" hypothesis (infection occurs before obstruction) and complementary hypotheses (SMG defects are pivotal).

To define early pathogenesis of CF lung disease, we will evaluate BAL from CF neonates, infants and children < 3 yrs of age . Two categories of patients will be studied. First, CF and non-CF patients undergoing bronchoscopy for clinical indications will be stratified on the basis of BAL analyses: 1) uninfected/uninflamed; 2) uninfected but inflamed; or 3) infected. Second, we will study neonates with meconium ileus in our effort to study infants at the earliest point in disease pathogenesis. Our goals are to measure the "mass of mucin" in BAL, characterize the cellular, bacterial and macromolecular content of mucus plugs, and characterize the ionic content and hydration of mucus plugs. To assist the reviewer, we will first describe patient groups, then bronchoscopy protocols, and assessment of inflammation and infection, and finally specific protocols.

a) Study subjects:

Infants and children undergoing bronchoscopy for clinical indications; (UNC Bronchology Center, **Director, Robert E. Wood, MD, PhD): We will focus on infants and children less** than 3 yrs of age (in 1996 at UNC, 234 children had clinically indicated BAL, including 24 CF patients <3 yrs of age (10 < 1 yr) and 108 non-CF children < 3 yrs. Indications for bronchoscopy in CF patients at UNC include a) newly diagnosed patients unable to expectorate sputum for culture, b) established CF patients who have lower airway symptoms unresponsive to therapy but unable to expectorate sputum c) chronic lobar atelectasis unresponsive to therapy. Non-CF patients with isolated laryngomalacia or tracheomalacia will serve as "normal" controls. Non-CF patients with aspiration, chronic atelectasis or recurrent pneumonia will serve as "disease" controls. Informed parental consent will be obtained. Over 90% of potential subjects at UNC participate in these studies. Patients receiving antibiotic therapy within 48 hours of bronchoscopy or chronic corticosteroid therapy will be excluded.

Neonates with meconium ileus: These patients will undergo bronchoscopy at the time of bowel surgery in the first few days of life. This novel study group will be particularly important for defining characteristics prior to any infectious or inflammatory insult. The majority of these patients will have CF, but definitive diagnosis of meconium ileus is not made until surgery; therefore, this population will probably include a few non-CF infants with ileal atresia or other congenital intestinal obstruction. To increase the number of eligible subjects (2-6 per year at UNC), we have enlisted collaborators at 4 major institutions in NC (Duke

Medical Center in Durham, Carolinas Medical Center in Charlotte, Mission Memorial Hospital in Asheville and Bowman Gray in Winston-Salem see attached letters). With this multicenter effort, we anticipate 10-20 patients per year. These infants will have a repeat study at 4-6 weeks of age when the colostomy is reanastomosed. Bronchoscopies will be performed by Drs. Barker, Leigh or Noah at UNC Hospitals and by collaborating pediatric pulmonologists at other institutions but attended by one of the co-investigators to ensure adherence to protocol.

b) General protocols/methods

Bronchoscopy and BAL: A standard pediatric bronchoscope will be introduced through the nose after appropriate sedation and positioning (discussed in Spec. Aim lB). The suction port will not be attached to suction until the time of BAL to avoid contamination with proximal airway secretions. After wedging the bronchoscope in the lingula, normal saline will be instilled by standard BAL protocol *(5* cc for infants < 1 month and 10 cc for infants> 1 month, followed by *1.5* cc of air to flush, immediate aspiration and then a second BAL of the same volume and location). In the clinically indicated bronchoscopy group, a site other than the lingula may be lavaged if indicated by clinical findings. From previous studies, 40-60% of instilled saline will be recovered in the BAL; sample recovery <40% will exclude samples. For all studies, we will record the status of airways inflammation (erythema, edema, increased friability or increased secretions).

Processing BAL fluid: Immediately after recovery, BAL volume will be measured and aliquoted for different analyses. We anticipate 4-6 ml from neonates and 8-12 ml from others. Immediately after recovery, 1.0 ml will be transferred into a vial containing 10 ml perflurocarbon for isolation of mucus plugs, *0.5* ml will be sent for culture, 450 il will be used for hemocytometer counts and preparation of 2 cytospin slides, and 3 ml applied to a cell strainer (70 pM nylon mesh) and centrifuged (1200 RPM for **S** mm) to separate mucus strands and plugs from supernatants and cells. Mucus trapped in the strainer will be eluted (300 ìl of 10 mM DTT) for analyses for mucin, DNA and F-actin. Cells in the pellet will be used for PMN studies. Aliquots of supernatants with and without filtration through 0.22 ìm filters will be frozen immediately at—80°C for cytokine, bactericidal activity and LPS assays. Up to *0.5* ml may be needed for additional clinical studies. The remaining BAL will be pipetted and frozen (—80°C). All samples will be coded, entered into the data log and placed in freezer boxes.

Assessment of infection: BAL fluid will be sent to the UNC Hospitals Microbiology Lab (Director, Peter Gilligan PhD) for quantitative bacterial culture and viral, mycobacterial and fungal cultures. The criterion for uninfected is < 50,000 cfu of bacteria/ml BAL. An aliquot of mucus plugs solubilized in 10 mM DTT will be cultured to quantitate viable bacteria in plugs. Uninfected BAL samples will be screened for LPS using a chromogenic Limulus amoebocyte lysate assay (60).

Assessment of inflammation: Total cell counts will be determined by hemocytometry immediately in the bronchscopy lab. Cell differentials will be determined by microscopic evaluation of 200 consecutive cells at high power on cytocentrifuge slides by an evaluator who is blinded to subject identity and diagnosis. The criterion for uninflamed is <10% neutrophils. IL-8 and IL-b concentrations in BAL will be assayed using specific, commercial ELISA kits (R&D Systems) as we have done previously (62).

SUBAIMS FOR SPECIFIC AIM 2B:

2B-1) Quantitate "mucin" and markers of SMG secretion in BAL to evaluate mucus stasis in uninflamed airways as well as mucus hypersecretion in infected airways.

1. **Rationale: Our** primary objective is to measure "mucin" content of BAL fluid as an estimate of intraluminal mucus in CF and non-CF neonates and infants with uninfected and uninflamed airways to determine if mucus is more abundant in CF airways. The overall mass of "mucin" recovered by lavage (estimated by quantitating PAS reactive material relative to a purified airway mucin standard) will be normalized to recovered BAL volume and to volume of sampled ASL using urea dilution correction. This semiquantitative approach will provide an overall index of mucin in the lavaged airways. In subjects exhibiting no inflammation or infection, we presume that increased "mucin" reflects mucus stasis and/or primary mucin hypersecretion. Lysozyme and lactoferrin will be used as markers of SMG secretion, but only for uninflamed samples because activated PMNs and macrophages may release these agents. We anticipate 3 potential outcomes. First, a significant increase in BAL "mucin" content in CF infants relative to non-CF controls, in the

absence of a significant increase in lysozyme or lactoferrin, will provide evidence for mucus stasis and not hypersecretion. Second, an increase in both mucin and lactoferrinllysozyme in the absence of inflammation will suggest primary hypersecretion. Finally, a decrease in lysozyme and lactoferrin in uninfectedluninflamed CF airways may reflect SMG dysfunction. To define the impact of decreased lysozyme and lactoferrin in the airways, we will measure their bacterial killing activity relative to the small salt-sensitive antimicrobials that have been emphasized by the hypotonic ASL hypothesis(8). Because lysozyme/lactoferrin assays are part of this protocol, and because questions about the role of decreased bactericidal activity are important regarding pathogenesis, we have developed assays to test antimicrobial activity for large (>10 kD) antimicrobials (including lysozyme and lactoferrin), vs small (<10 kD) antimicrobials (including defensins).

Likewise, we will quantitate "mucin" in BAL from "infected" subjects to determine if mucus is increased relative to comparable non-CF subgroups and increased relative to the uninfectedluninflamed CF subgroup. Bacterial products and inflammatory mediators are known to stimulate mucin secretion from SMGs and goblet cells (10 1-106); and, we expect a further increase in mucus consequent to infection and inflammation in both the CF and non-CF groups. DNA and F-actin contents should be minimal in uninfected and uninflamed airways, but increase markedly during infection and inflammation, and may contribute further to mucus stasis and plug formation. We will assess the relative amounts of mucin, DNA and F-actin in BAL fluid and in mucus plugs (subaim 2) to better determine their role in mucus plug formation.

The uninfected but inflamed group is particularly intriguing. It is difficult to interpret the significance of inflammation without apparent infection, because the possibility of previous infection cannot be excluded (62). Careful monitoring of the meconium ileus infants during the interval between surgeries will be important for this issue. Consistent with our hypothesis, we propose that dehydrated mucus itself or bacteria enmeshed in mucus plugs but not recovered on routine bacterial cultures, serve as inflammatory stimuli to attract PMNs into the airway. To evaluate the latter, we will culture solubilized mucus plugs, assay BAL for LPS, and examine plugs by light and TEM for bacteria (see Subaim 2). Another possibility is that routine lung insults (such as exposure to tobacco smoke) elicit an amplified or prolonged PMN infiltration in CF airways. An imbalance of proinflammatory (JL-8) and inhibitory (IL-b) cytokines in CF airways (63) may amplify inflammatory responses; therefore, we will analyze cytokine profiles obtained as part of patient characterization. Alternatively, the lifespan of PMNs in airway lumen may be prolonged in CF, e.g., via delayed apoptosis leading to an apparent PMN excess in CF airways. Bacterial products such as LPS may impair PMN apoptosis (107); therefore, we will assess PMN apoptosis and LPS in inflamed but uninfected BAL samples.

2,3. Specific methods/protocols:

a) Measure "mucin": PAS reactive glycoconjugates in BAL will be measured using the PAS slot blot analysis described in Spec Aim 2A. A 10 ìl aliquot of solubilized mucus (strained from 3 ml BAL and solubilized in 300 ìl DTT) will be used for initial testing. The volume will be increased (10-20 fold) if necessary. PAS reactive material will be measured relative to a standard, purified CF mucin to yield mucin equivalents in ìg/ml and we will calculate ìg of mucin equivalents per ml BAL fluid and ìg of mucin equivalents recovered in the total BAL volume. Finally, ìg of mucin equivalents/mb ASL will be determined from urea ASL dilution referenced to serum urea (62,108). BAL samples will also be assayed by ELISA using an antibody (H6C5) against respiratory mucin (described in Spec. Aim 2A) to detect mucin antigen equivalents. This ELISA is 10 fold more sensitive than the PAS assay (see Prelim. Data), and may be important for neonatal BALs that may contain minimal mucin. In these small samples, extensive isolation and purification of mucins will not be possible. If the PAS analyses indicate that adequate material is present, we will isolate hyaluronidase-resistant, high molecular weight glycoconjugates (HMWG) by Sepharose 4B-CL chromatography following bovine testicular hyaluronidase digestion of glycosaminoglycans, using established protocols (80,109,110). These HMWGs will be quantitated by slot blot analysis of PAS reactive material and mucin ELISA.

b) Lysozyme and lactoferrin activity -absolute and relative to smaller antimicrobials: These parameters will be assayed in "uninfected/uninflamed" BALs. Briefly, lysozyme activity will be assayed from bacterial cell wall hydrolysis relative to lysozyme standard; and lactoferrin will be quantitated using an ELISA kit (as described in Spec Aim 2A). The values will be normalized to total BAL volume and to ASL volume as

for “mucin”. Antibacterial activity of these large (>1 OkD) antimicrobials will be assessed and compared with that for smaller (<10 kD) antimicrobials, including defensins. BAL supernatants that have been passed through 0.22 ìm filters and frozen at—80°C will be divided into fractions <or> 10 kD by filtration through spin columns with 1 OkD cutoff (Amicon). Standard solution bactericidal assays (111) will be performed using *E. coli* or clinically relevant bacteria *(P. aeruginosa, S. aureus, H influenzae* obtained from P Gilligan, UNC).

c) DNA and F-actin in all BAL samples will be assayed by standard protocols. The DNA content will be quantified relative to calf thymus DNA standard by ethidium bromide staining after immobilization on nylon membranes using standardized procedures (sensitivity <100 ng) (112). Microfilamentous F-actin is quantified by a modified DNase inhibition assay (113,114) which is based on the inhibition of DNase I digestion of DNA by G actin. Briefly, the fluorescent dye, TO, is used to measure the DNA (using a fluorescent plate reader) remaining in microtiter wells after incubating DNA with a mixture of DNase and actin. Purified rabbit muscle actin (from Cytoskeleton, Denver, CO) will be used to construct the standard curve. Because the assay uses exogenous DNA at very high concentration, interference from endogenous DNA in BAL samples will be minimal.

d) To characterize PMNs in the “uninfected but inflamed” subgroups, the prevalence of apoptotic PMNs will be determined by counting the number of apoptotic PMNs per 200 PMNs on each cytospin slide stained with a modified Wright’s stain. Apoptotic PMNs have recognizable morphologic features by light microscopy (66,67).

4. Data Analysis/Limitations: We will compare “mucin” content (expressed both as ìg of mucin equivalents in total lavage and as ìg of mucin equivalents/mb ASL) in “uninfected and uninflamed” CF infants versus age matched “uninfected and uninflamed” controls (infants with isolated larygomalacia or tracheomalacia). Then, we will determine if “mucin” content increases with age of infants. An age-related increase in “mucin” content in CF infants relative to controls in the absence of infection and inflammation would support the notion of mucus stasis with progressive accumulation if lysozyme and lactoferrin remain constant. Alternatively, a significant increase in CF BAL lysozyme/lactoferrin would support primary hypersecretion; whereas a significant decrease in lysozyme/lactoferrin would support SMG dysfunction.

A key issue is our approach to quantitate “mucin”. Isolation of airway mucins involves multiple purification procedures (115-117). The relatively small size of our BAL samples limits our ability to purify mucins. We adapted a simple assay of PAS reactive material for measurements in BAL (Prelim. Data). While this assay is not specific for mucins, we expect most of the PAS-reactive material in BAL should be mucins, because these samples should have minimal amounts of glycogen. Isolation of hyaluronidase-resistant, HMWG eliminates glycogen and most of the glycoconjugates that may react with PAS and H6C5 mAb, and therefore, will optimize our assays. During the period of this grant, we plan to develop assays using specific antibodies generated against peptide regions of MUC 1, MUC 2, MUC SAC, MUC SB and MUC7 to more specifically quantitate mucins (see letter from S Randell Ph.D.). These efforts will include evaluation for potential loss of specific epitopes due to mucin peptide hydrolysis by proteinases, because infected and inflamed BALs contain proteinases. Initially, we will assay “mucins” by 2 methods (PAS slot blot and ELISA). A consistent and parallel quantitation of mucin using these 2 detection systems will confirm that these assays are not confounded by modification of specific epitopes (e.g., by glycosidases and/or proteinases in infected airways). A limitation of this protocol is the restriction to patients undergoing bronchoscopy for clinical indications, thereby excluding true normal controls. Research bronchoscopy in pediatric patients unlikely to derive benefit from the procedure (e.g. normal children) is considered inappropriate at most institutions. Our control population is heterogeneous and includes children with different underlying conditions with and without infection. We have addressed the limitation of small numbers of meconium ileus patients through collaboration with other institutions providing surgical treatment for infants with meconium ileus. Several of these collaborators received their training at UNC and have a strong allegiance. Over the 5 years of this grant, we should have the opportunity to study 25-40 meconium ileus infants (of which 20-35 should have CF).

2B-2) Characterize the cellular and macromolecular composition of “mucus plugs”

1. Rationale: Mucus plugs are small (<3 mm) aggregates of mucus that are visibly suspended in BAL fluid. While BAL recovers mucus covering a broad region of airways, mucus plugs reflect changes in a

localized region. A detailed morphologic characterization of plugs defining presence (or absence) of mucin, bacteria, PMNs, DNA and actin will be used to classify plugs based on composition, i.e., 1) mucin with few PMNs, little actin or DNA and no bacteria, 2) mucin with many PMNs (and PMN products e.g., DNA and actin) but no bacteria, 3) mucin with many PMNs and bacteria, and possibly 4) mucin and bacteria but few if any PMNs, actin or DNA. Our goals are to determine if mucus plugging occurs prior to infection and inflammation and to characterize the composition of these plugs relative to the state of infection and inflammation in surrounding BAL. If isotonic volume absorption is correct, then dehydrated mucin should form plugs in airways prior to infection and inflammation.

2,3. Specific methods/protocol: BAL fluid (1 ml) will be immediately mixed with 10 ml perflurocarbon (PFC) (FC-72 Fluorinert, 3M). The aqueous phase does not mix with PFC and immediately separates to form a layer on top of the dense PFC. This aqueous layer will be aspirated, leaving the mucus plugs suspended at the upper surface of the PFC, which allows collection for morphologic studies (see Prelim. Data). For light microscopy, isolated plugs are transferred from PFC to OCT, frozen, and sectioned for H&E, PAS (mucin), TO-PRO- 1 iodide (DNA), and TRITC labeled phalboidin (F-actin) (118). We have successfully stained for these components in mucus plugs (Prelim. Data). We are adapting methods to stain with PAS, TO-PRO and phalloidin on the same slide. Using computer-assisted morphometric analysis, we will measure the diameter and cross-sectional area of each mucus plug in the frozen sections. Then, we will determine the specific area of plug cross-sections that is composed of mucin, DNA and F-actin. A ratio of the F-actin or DNA area to mucin area should reflect the relative amounts of these macromolecules in plugs. These ratios will be compared with complementary ratios from biochemical analyses in Subaim 1. For ultrastructural analyses, mucus plugs will be fixed in 1% OsO_4 in PFC, dehydrated and infiltrated with epon by the protocol used to preserve tracheal mucus (119). To further characterize the bacteria within plugs, we will use routine bacterial stains as well as immunostaining with antibodies to *P. aeruginosa* and *S. aureus.* Also, we will solubilize mucus plugs in 10 mM DTT and culture by routine methods used for unmodified BAL to determine if viable bacteria are enmeshed in plugs. By transmission electron microscopy, we will count the number of bacteria and PMNs in 20 non-overlapping fields examined at a magnification of 1 0,000X to determine their prevalence. In addition, PMNs will be evaluated for ultrastructural features of apoptosis and necrosis.

4. Data analysis/limitations: Based on our central hypothesis, we expect to identify some plugs from "uninfected and uninflamed" patients containing mucin and minimal (if any) F-actin and DNA. Once infection is established, we predict that plugs will reflect the combined influences of dehydration, increased mucin secretion, and increased concentrations of DNA and F-actin. By careful analysis of the mucus plugs for components thought to arise from neutrophils (F-actin and DNA), we will be able to determine the relative importance of inflammation. We will compare the F-actin/mucin (or DNA/mucin) ratios for the "uninfected and uninflamed" CF group to the other two CF subgroups to determine if the relative amounts of these other macromolecules are lower in the absence of inflammation. In non-CF patients, mucus plugs likely result from inflammatory or infectious insults that cause epithelial damage resulting in mucus stasis, mucus hypersecretion and PMN infiltration contributing additional DNA and actin to the mucus gel. While we may not observe mucus plugs in the "uninfected and uninflamed" non-CF group, comparisons between CF and non-CF for the "infected" and "uninfected but inflamed" may identify compositional differences.

Defining the airway source of mucus plugs is complicated. Mucus plugs may partially fill the airway 'umen; therefore plug diameter does not necessarily equal airway diameter. Our mucus plug measurements will be used to estimate a lower limit for size of the plugged airway. Swelling of mucus plugs during transient exposure to saline may bias our estimate toward a larger size. Hydration of mucus during exposure to saline will be addressed below. Ultimately we plan to use PFC for lavage to minimize changes in plug size.

One limitation is the difficulty excluding the possibility of prior infection in patients with plugs but no active infection. We will use several approaches to test for bacteria in plugs, including histologic and immunostaiing for bacteria, bacterial culture of solubilized plugs, and TEM examination. We have experience with PCR for microbial agents (120) and considered PCR analysis here; however, small amounts of nasopharygeal flora may contaminate our bronchoscope during its insertion through the nose. Consequently, the sensitive PCR assay may detect transcripts from these nasal bacteria, leading to a false positive.

2B -3) Measure state of hydration and ion content of "mucus plugs"

1. Rationale: The composition of mucus plugs recovered from airway lavage will characterize the nature of the obstructing element in CF airways, and if the plug is in equilibrium with the surrounding airway surface, will characterize the composition of ASL in small airways beyond access of filter paper sampling (see Spec. Aim 1B). The key to this protocol is to harvest plugs without perturbing their ionic or water composition. Two approaches are proposed. First, we will analyze ionic and water content of the plugs that are currently harvested by saline BAL. The current protocol entails rapid transfer of plugs from BAL to water immiscible perfluorocarbon (PFC). This approach has the advantages of being practical and the samples are available. If CF plugs are "dehydrated", relative to non-CF, it would be important information. However, we are very concerned about the possibility of ion/water redistribution during harvesting with saline BAL. We will test for the magnitude of this redistribution by exposing mucus of defined ion/water composition to saline after direct harvest from our well-differentiated epithelial cultures (see Project I). Secondly, we are developing a PFC lavage protocol which would be ideal for harvesting plugs in this project. PFC is a water and mucus immiscible liquid that has been used for liquid ventilation in premature infants (73).

2,3. **Specific methods/protocol: For our** current approach, we use fine forceps to transfer individual mucus plugs from PFC to an aluminum pan and weigh immediately to obtain a wet wgt (see Methods in Spec. Aim 1A). The mucus plugs are dried (1000 C for 24-72 hours until wgt stabilizes) to obtain a dry wgt, and permit calculation of% dry wgt (dry wgtlwet wgt x 100). Ions (Na^+, Cl^-, K^+, Ca^{2+} and Mg^{2+}) will be analyzed by the Analytic Core.

4. Data analysis/limitations: We will measure wet and dry wgts, and calculate % solids and ion composition of mucus plugs to determine the state of hydration and tonicity of mucus in distal airways. The plugs from CF patients who are "uninfected and uninflamed" will be the most informative, since they will have no added solids from inflammatory cells or epithelial shedding to confound the dry/wet wgt ratio. We will compare the % solids and tonicity of those CF plugs to any available non-CF plugs that are "uninfected and uninflamed", and to the % solids (and water content) of filter paper samples from "uninfected and uninflamed" non-CF infants or young children (Spec. Aim 1B). We will also compare the % solids of plugs from the other CF subgroups ("uninfected and inflamed" and "infected and inflamed") to their respective non-CF "control" groups. With an optimal technique to harvest plugs, we predict that we would see increased % dry wgt in CF plugs as compared to non-CF plugs or non-CF filter paper samples, but the saline BAL technique may not allow detection of such possible differences.

The limitations of this protocol relate to the method of obtaining plugs and the availability of pertinent disease controls. It is well-recognized some non-CF infants/children have plugs on BAL, and we are confident that the large number of patients requiring bronchoscopy at UNC will provide plugs from non-CF infants and children with a range of infectious and non-infectious pulmonary disease. It is possible that we will get few (or no) "uninfected and uninflamed" mucus plugs from non-CF controls. If so, we will compare % solids from "uninfected and uninflamed" CF plugs to the % solids of filter paper samples from ipratropium-pretreated (to block possible SMG secretion) non-CF controls who are "uninfected and uninflamed" (see Project IB). We currently obtain mucus plugs by saline BAL and rapidly transfer into water-immiscible PFC, which allows ~xcess surface liquid to be separated from the plugs. The transient (< 5 mm) exposure of plugs to saline may significantly perturb the water (and ion) content. We definitely recognize this potentially significant limitation in the current protocol, and we plan to better define the effects of transient saline exposure. Specifically, we will expose mucus "plugs" from the surface of differentiated airway epithelial cell cultures grown at an air-liquid interface (acquired from Project I and the Tissue Culture Core) to saline for intervals between 0 and 5 minutes, and remove aliquots of mucus for transfer to PFC every minute. Each sample is processed (as above) for wet and dry weights and ionic composition. From this time curve, we will determine the relationship between the interval of saline exposure and change in hydration and/or ion composition. In parallel, we are emphasizing the development of other approaches, including BAL with glucose (non-electrolyte/isosmotic) or PFC. The latter vehicle would be optimal for the BAL collection of plugs to test for state of hydration and ion contents of distal airways to answer critical questions about CF pathophysiology.

e. HUMAN SUBJECTS

One aspect of this project will be performed at collaborating sites. Specifically, the study of airway surface liquid in infants born with meconium ileus will be performed not only at UNC, but also with collaborators at four other sites; Mission Memorial Hospital Complex, Asheville (Clarke McIntosh, M.D.), Carolina Medical Center, Charlotte (William Ashe, M.D. and Hugh Black, M.D.), Duke Medical Center, Durham (Tom Murphy, M.D.) and Bowman Gray Medical Center, Winston-Salem (Michael Schechter, M.D.). Each of these collaborators is a Board-certified or Board-eligible Pediatric pulmonary physician with extensive experience at bronchoscopy studies, and the Human Rights protocols are currently being processed by our collaborators at each individual site. We estimate that we will study 2 subjects per year at each institution. The demographics of study subjects at these other institutions will reflect the demographics of cystic fibrosis, since almost all infants with meconium ileus have CF. Specifically, *95%* of these patients will be of Caucasian or Hispanic extraction, reflecting the population distribution in North Carolina. We anticipate that the gender distribution will be equal between males and females. The other specific issues related to these studies will be addressed below.

(1) Study Subjects

a) Meconium ileus subjects: We anticipate studying 10 subjects per year, with follow-up studies in each subject at 1 to 3 months of age at the time of surgical take-down of ostomy site. We anticipate the gender will be distributed equally between male and female, and that 90% of these subjects will be of Caucasian extraction, and *5%* of Hispanic extraction, with a few % being African-American extraction. The major exclusion criterion will be clinical instability, which will be determined by physicians providing primary care for these neonates, which will not be the participating investigators.

b) Pediatric: We anticipate studying 30-35 CF and 30-35 non-CF patients per year, with the age range between birth and 3 years. The gender distribution will be equal between male and female, for both groups. For CF patients, the vast majority will be Caucasian, with a few patients of Hispanic or African-American extraction, which reflects the demographics of the genetic disease, cystic fibrosis. For the non-CF patients, the racial distribution will reflect the population of North Carolina, i.e., 20% African-American, 75% Caucasian, and *5%* Hispanic extraction. Inclusion criteria will be subjects who require clinically indicated bronchoscopy and BAL; roughly 2/3 of these subjects will suffer from aspiration or anatomic airway obstruction, and the remainder will have recurrent pneumonias, or unexplained persistent wheezing, or pulmonary disease of ill-defined etiology. We will not exclude anyone from this study on the basis of race/ethnic/gender considerations.

c) Adults: Over the five-year period of this clinical research project, we anticipate studying an estimated 160 adult subjects, some of them on more than one occasion. The breakdown of the estimated number of study subjects is: normal subjects (50), CF patients (50), patients with Sjögren's syndrome (10-12), patients with primary ciliary dyskinesia (PCD; 10-12), patients with pseudohypoaldosteronism (PHA) (6), and chronic rhinosinusitis (32). These study subjects will be 18 years or older, except for a few adolescent CF patients and some adolescents with PHA. There will be an equal distribution of male and female study subjects, but the racial/ethnic composition of the study populations will reflect the demographics of the disease, i.e., our CF patient population at UNC reflects the genetic predisposition of this specific disease, i.e., less than 5% of the CF patients have an ethnic background other than Caucasian, but the normal volunteer and other disease groups will reflect the population of North Carolina, i.e., 20% African-American, 75% Caucasian, 5% Hispanic.. We will not exclude anyone from the study on the basis of race/ethnic/gender considerations.

2) The research material obtained from each of these study subject groups will be in the form of nasal secretions or bronchoalveolar lavage fluid, except in the case where blood is drawn for urea determination to allow for correction for volume sampled. Medical records will be reviewed as per clinical indications. For studies in neonates and children, only the BAL from meconium ileus study subjects and blood samples for urea are obtained only for research purposes; all other materials are obtained for clinically indicated procedures. In the adult study populations, all material specimens and data will obtained specifically for

research purposes. There are no existing specimens or data that bear directly on addressing the current research questions.

(3) Neonatal patients undergoing bronchoscopy will be surveyed by collaborating investigators for presentation of meconium ileus, and the ongoing studies of children undergoing clinically indicated bronchoscopies will be surveyed by the weekly meeting of the Pediatric Pulmonary group. For the studies performed at collaborating sites, the collaborative investigators will obtain permission under the auspices of the local institutional IRB guidelines. Recruitment of adult study subjects and disease-control groups will be undertaken by the P.1. or one of the participating investigators, and informed consent will be obtained under the guidelines of the UNC IRB.

(4) Regarding potential risks for the meconium ileus studies, these largely involve inserting the bronchoscope and performing BAL in a neonate who is intubated and under general anesthesia. These risks may include minor trauma to the airway with possibilities of minor bleeding or cardiac arrhythmias, as well as a risk of venipuncture (if we are unable to get blood from the I.V. site). For the research involving clinically indicated bronchoscopies, the only additional risk factor is drawing of blood for urea determination for correction of volume of ASL sampled. For the adult studies involving nasal and bronchial airway surface liquid, the risks are relatively few. Specifically, the only complication we have noted with nasal studies over the last 10 years is an occasional blood tingeing of nasal secretions, or occasional (4-6) episodes of vasovagal reaction, which had been treated by standard techniques without significant long-term complications. The bronchoscopy studies in volunteers and disease-control subjects carry the usual risks of bronchoscopy, which are a low incidence of largyngospasm, cardiac arrhythmias, or minor trauma to the airway wall. To date, we have not had a significant adverse event associated with these research procedures.

(5) We undertake all possible actions to protect against potential risks in our human studies. Regarding the meconium ileus patients, we will study only patients that are stable and being intensively monitored by an anesthesiologist, and the BAL is performed by professionals experienced at performing bronchoscopy, and only under IRB-approved protocols. The research risks in the pediatric patients undergoing clinically indicated bronchoscopy are none, except for the possibility of a hematoma by blood drawing, which can be addressed by adding pressure, and the potential loss of confidentiality, which is addressed by coding samples by number rather than by name. Regarding the studies of adults, we monitor all patients and normal volunteers undergoing bronchoscopy with cardiac and oxygen saturation monitors, and have specific personnel only for that purpose. Our bronchoscopies are performed by experienced personnel in a standard hospital bronchoscopy suite setting. For nasal studies, we screen carefully for history of drug allergy and fully inform the patient of the procedures to be undertaken.

(6) Regarding the risk/benefit to subjects undergoing these research procedures, there is a clear potential benefit for the infants born with meconium ileus, in view of the high likelihood that they suffer from CF, and they may benefit from studies that define the basic early pathophysiology of lung disease. Likewise, similar benefits are available for the CF patients undergoing clinically indicated bronchoscopies. The only risks for pediatric patients undergoing clinically indicated bronchoscopies are the possibilities of hematoma and confidentiality issues. These studies are likely to pay dividends in terms of defining the early pathogenesis of CF lung disease, as well as providing us with insights into the role of airway epithelial ion transport function and the role of ion composition in airway defense. For the adult volunteers, the risks are small but finite, and seem reasonable in view of the importance of defining the normal role of ion composition and local antimicrobial and airway defense mechanisms. There are no new drugs being tested in this protocol.

f. VERTEBRATE ANIMALS

N/A

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h. CONSORTIUM/CONTRACTUAL ARRANGEMENTS

1. N/A

2. Describe the collaborations of investigators within the SCOR

Project IV focuses on a central SCOR theme, that is, discriminating between the isotonic volume hypothesis and the hypotonic airway surface liquid hypothesis by testing these hypotheses in cystic fibrosis infants prior to and early in the initiation of CF lung disease. Thus, Project IV has heavy interactions with Project I, which focuses on similar themes. Specifically, comparisons will be made with respect to the composition of airway surface liquids from the *in vitro* preparations studied in Project I with those measured *in vivo* in Project IV. Similarly, the effects of potentially raised volume absorption on mucus water content (% solids) will be compared to those derived from Project IV. The solutions recovered from airway surfaces under basal conditions presumably reflect the activities of the CFTR protein under resting conditions, i.e., resting CF conductance and the putative resting CFTR-dependent regulation of Na^+ transport (ENaC). Thus, interpretation of the data derived from these studies in Project IV rests upon the mechanistic studies derived in Project II with regard to the topographical localization and regulation of CFTR activities by membrane-associated PKA holo-enzymes and anchoring proteins (AKAPs). Finally, a theme of Project IV is to understand the balance in proximal airways between secretion (presumably a glandular function) and absorption (we speculate a superficial epithelial function). Thus, Project IV interfaces with Project III, which is designed to elucidate sites of secretion in the murine lung and block these activities using the gene targeting approach, focused on NKCC 1. The most direct comparison will be in the putative composition and regulation of gland secretions in the proximal murine trachea and our studies in proximal human airways.

Project IV relies heavily on the Core activities of the Analytic Core and the Morphology Core. A critical issue in the evaluation of the competing hypotheses is the composition of airway surface liquids. Thus, we will rely heavily on the Analytic Core to generate data that relate to the ionic content of harvested airway secretions, osmolality, and divalent ion concentrations. A key hypothesis of Project IV is that excessive volume absorption will desiccate airway mucins, leading to retention and airway plugging. Thus, Project IV will rely heavily on the Morphology Core for the assessment of the presence or absence of plugs and compositional analyses of plugs vis-à-vis mucins, actins, and cellular contents.

i. CONSULTANTS

N/A

j. Discussion of Anticipated Results

We have detailed the specific results expected for each Specific Aim and Sub-Aim in the Experimental Design and Methods section (see Data Analysis/Limitations), and how the data will be analyzed and interpreted. We think the research design of our protocols will enable us to confirm (or refute) our major hypothesis, and also allow us to determine the validity of several alternative hypotheses.

In the overview, this proposal is an ambitious, state-of-the-art approach to clinical research involving the study of pediatric patients (including neonates) and the application of novel methodologies (including ion-selective electrodes, *in vivo).* We recognize the challenges of such a project, but believe that the important questions being asked about CF pathophysiology, coupled to the strength of the clinical and research

investigators and technical resources make it highly likely that important physiological (and pathophysiological) concepts will emerge from this project. The research team is experienced and has a track record of productive interaction, including the development of innovative approaches applied to the study of human subjects, *in vivo*.

The major questions for this project, and anticipated outcomes, are summarized below:

1) What is the ion composition of airway surface liquid (ASL) in normal airways? We expect to definitively answer whether normal ASL is isotonic or hypotonic. We anticipate that normal airways will have isotonic ASL under basal (unperturbed) conditions, which suggests that airway epithelia are relatively water permeable, and exhibit isotonic volume absorption. The outcome of these measurements has broad implications for the overall understanding of airway liquid metabolism and the role of salt-sensitive antimicrobials in airway defense.

2) What is the relative contribution of surface epithelia versus submucosal gland (SMG) epithelia to the net balance of ASL ion composition? We expect to confirm that surface epithelia absorb NaC1 and volume, and SMGs secrete a hypotonic fluid. Taken together, these outcomes will provide a clearer view of the "local" (SMG versus surface epithelia) metabolism of ASL, and provides strong indirect evidence for a water-permeable surface epithelium and the "isotonic volume absorption" hypothesis.

3) Does the ion composition (including pH) of CF ASL differ from normal? If so, does it reflect dysfunction of the surface and/or SMG epithelia? We expect to find that the tonicity of CF ASL is not different from normal, but the surface epithelia may transport anions differently from normal, and thereby alter pH and/or HCO_3 of ASL. We expect to find that CF SMGs have a major non-CFTR mediated secretory pathway that drives the production of normal volumes of glandular secretion. However, the ionic composition of CF SMGs may differ from normal, reflecting a slightly larger than normal secretion of an anion other than CF (perhaps HCO;). Clarification of the differences between ASL ion composition in CF versus normal has critical importance for understanding the pathophysiology, and targeting therapy, toward the primary epithelial dysfunction in CF airways (see below).

4) What is the initial event in the pathogenesis of CF airways disease, and how does it link to abnormal ion transport? The answer to this question is complex, but likely relates to the key outcomes of our experimental protocol. We expect to find that normal airways exhibit isotonic volume absorption (see above), which implies that CF airways disease does not result from the inhibition of salt-sensitive small-molecule antimicrobial activity. Isotonic volume absorption in normal airways also implies that excessive volume is absorbed in CF airways, and poor hydration of mucin likely lies at the root of the problem of viscous mucus in CF. The latter may be further complicated by changes in ASL pH, or HCO_3^- (concentration, reflecting altered anion transport by surface epithelia and/or SMG epithelia. This project is designed to test the consequences of these abnormalities on airway mucus, and we expect to demonstrate that abnormal mucus hydration results in accumulation of mucus and initiation of airways disease in CF. In that regard, our studies in neonates with meconium ileus are likely to answer the seminal question, i.e. do abnormal airway secretions precede infection and inflammation, or does infection or inflammation occur first?

The outcomes of the clinical research studies in Project IV address the broad theme of the SCOR proposal, and will be interpreted in the context of ongoing complementary studies in Projects I, II, and III. We believe that the studies in Project IV will provide insight into normal physiology of airway surface liquid metabolism, and better understanding of the link between mutations in CFTR and pathogenesis of airways disease.

k. Significance

The significance of Project IV relates directly to the overall theme of the SCOR, which is focused on the pathogenesis of CF lung disease. Project IV has two major components: first, *in vivo* studies designed to generate a better understanding of the metabolism of airway surface liquids in normal human airways, and integrating the role of surface and submucosal gland epithelial ion (and volume) transport, and mucus hydration. The second component involves direct studies of CF infants and neonates and age-matched disease control patients, to define the initiating event(s) in the early pathogenesis of CF lung disease. The outcome of this research effort has direct clinical applications. Better definition of the nature of the defects that contribute to the early pathophysiology of CF airway disease will improve the opportunity to develop targeted new therapies,

and identify appropriate surrogate markers that will speed the progress of clinical studies of new therapeutic agents in CF.

1. Facilities Available

The faculty and personnel in Project IV have contiguous lab and office space, which is geographically adjacent to UNC Hospitals and the General Clinical Research Center. The physical facilities and space are detailed in Form Page HH (see p. 68). Project IV also takes advantage of the large CF patient population and disease control patients available at UNC. These are discussed in detail in section “e” (Human Subjects).